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PARASITOLOGY

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LIVERPOOL SCHOOL OF TROPICAL MEDICINE

PATRON: HIS MAJESTY THE KING

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CONTENTS

No. 1. April, 1948

	PAGE
ADLER, S. ; TCHERNOMORETZ, I. ; and BER, M. The action <i>in vitro</i> of some aromatic diamidines on a Sudan strain of <i>Leishmania infantum</i>	1
KETTLE, D. S. The growth of <i>Anopheles sergenti</i> Theobald (Diptera, Culicidae), with special reference to the growth of the anal papillae in varying salinities	5
NASH, T. A. M. A note on the effect of high temperature on the pupal stage of <i>Glossina</i> in relation to the transmission-rate of trypanosomes	30
STEWART, G. T. ; and JONES, W. R. The pathology of an experimental amoebic infection in the rat	33
BLAIR, D. M. ; and ROSS, W. F. Observations on the use of cercarial antigen in the diagnosis of schistosomiasis	46
BLACK, R. H. Leishman's stain adapted for use with histological sections	52
GALL, D. A simple technique for the microscopy of living tissues <i>in situ</i> , with some observations on the splenic circulation	54
MACFIE, J. W. S. Some species of <i>Culicoides</i> (Diptera, Ceratopogonidae) from the State of Chiapas, Mexico	67
GRAMICCIA, G. ; and BLACK, R. H. The cultivation of exoerythrocytic forms of <i>Plasmodium gallinaceum</i> . I : A preliminary note	88
BLACK, R. H. The resorption of haemoglobin by the renal tubules in haemoglobinuria	90
ANDREWS, W. H. H. ; and MAEGRAITH, B. G. The pathogenesis of the liver lesion due to the administration of carbon tetrachloride	95
CHWATT, L. J. Infection of reticulocytes by <i>Plasmodium falciparum</i> and <i>Plasmodium malariae</i> in hyper-endemic indigenous malaria	101
COGHILL, N. F. ; and GAMBLES, R. M. Discussion of methods for differentiating tick- from louse-borne relapsing fever spirochaetes	113
MISCELLANEA	118

CONTENTS

No. 2. September, 1948

	PAGE
COWPER, S. G. The effect of certain inorganic and vegetable substances on the English pond snail <i>Planorbis</i> <i>corneus</i> (Linné, 1758)	119
VANDERPLANK, F. L. Experiments in cross-breeding tsetse-flies (<i>Glossina</i> species)	131
FOY, H.; KONDI, A.; DAMKAS, C.; DEPANIAN, M.; LEFCOPOULOU, T.; BACH, L. G.; DAX, R.; PITCHFORD, J.; SHIELE, P.; and LANGTON, M. Malaria and blackwater fever in Macedonia and Thrace in relation to DDT	153
PAWAN, J. L. Fruit-eating bats and paralytic rabies in Trinidad	173
ADLER, S.; and ZUCKERMAN, A. Observations on a strain of <i>Leishmania tropica</i> after prolonged cultivation: notes on infectivity and immunity	178
CHWATT, L. J. A new <i>Aedes</i> from the Cameroons, <i>A. (Aëdimorphus) boneti</i> s.sp. <i>kumbae</i> s.sp.nov. ...	184
SPINKS, A. Studies on synthetic antimalarial drugs. XX: The blood concentrations and physiological distribution of some homologues of paludrine in relation to their antimalarial activities	190
STEWART, G. T. The rôle of bacteria in intestinal amoebiasis in man	198
DAVIS, D. H. S. Sylvatic plague in South Africa: history of plague in man, 1919-43	207
HADDOW, A. J.; SMITHBURN, K. C.; DICK, G. W. A.; KITCHEN, S. F.; and LUMSDEN, W. H. R. Implication of the mosquito <i>Aedes (Stegomyia) africanus</i> Theobald in the forest cycle of yellow fever in Uganda	218
CORKILL, N. L. Activation of latent kala-azar and malaria by battle experience	224
CORKILL, N. L. Activation of latent kala-azar by malaria and relapsing fever	230
BLACK, R. H. Haemoglobin stains adapted for use with thin blood films	236
WILLIAMSON, J. A nomogram for the preparation of standard inocula in the Davey chick test of antimalarial activity... ..	238
ROLLO, I. M.; WILLIAMSON, J.; and LOURIE, E. M. Acquired paludrine-resistance in <i>Plasmodium gallinaceum</i> . II: Failure to produce such resistance by prolonged treatment of latent infections	241
UNSWORTH, K. Observations on the occurrence of larvae of <i>Oestrus ovis</i> in the nasal cavities and frontal sinuses of goats in Nigeria	249
MISCELLANEA	251

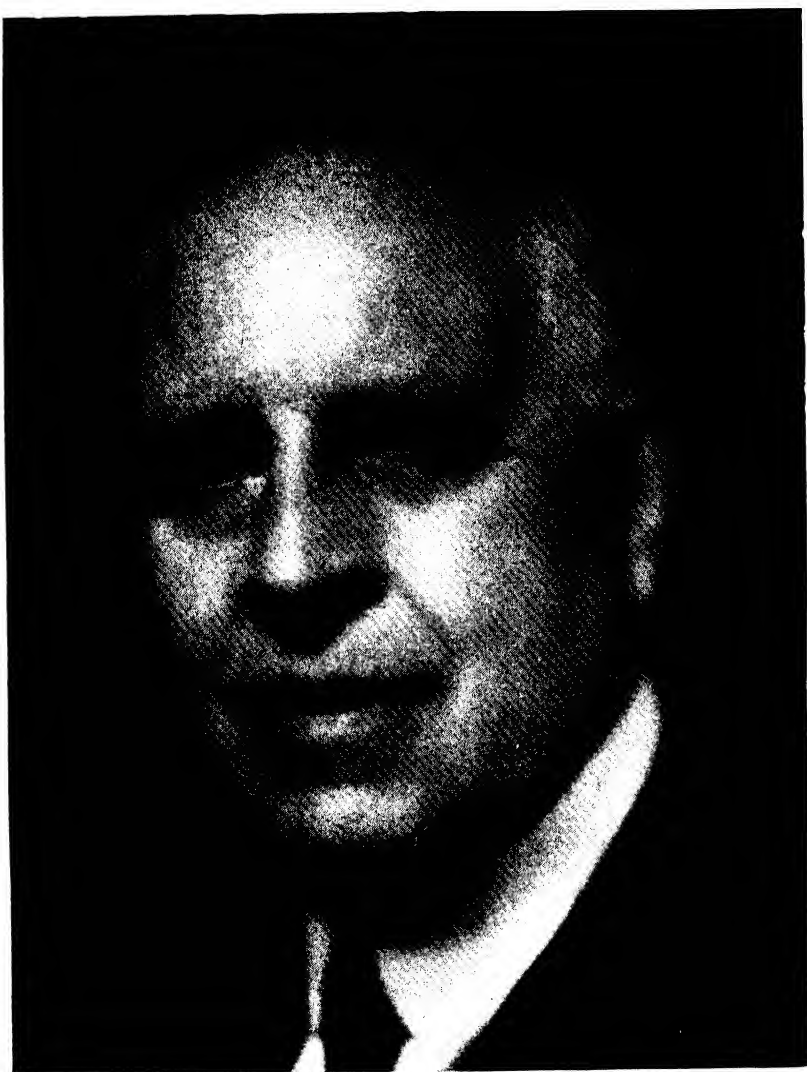
CONTENTS

Nos. 3 and 4. December, 1948

	PAGE
LEESON, H. S. Anopheline larvae collected in Arabia	253
KIKUTH, W. ; and GÖNNERT, R. Experimental studies on the therapy of schistosomiasis	256
VOGEL, H. ; and MINNING, W. The action of miracil in <i>Schistosoma japonicum</i> infections in laboratory animals	268
HADDOW, A. J. ; and DICK, G. W. A. Catches of biting Diptera in Uganda, with anaesthetized monkeys as bait	271
CORKILL, N. L. The poisonous wild cluster yam, <i>Dioscorea dumetorum</i> Pax, as a famine food in the Anglo-Egyptian Sudan	278
GAMBLES, R. M. ; and COGHILL, N. F. Relapsing fever in Cyprus	288
HALAWANI, A. ; BAZ, I. ; and MORCOS, F. Field trials with new antimalarial drugs in Egypt	304
CARTER, H. F. Records of filaria infections in mosquitoes in Ceylon... ..	312
KIRK, R. ; and LEWIS, D. J. Taxonomy of the Ethiopian sandflies (<i>Phlebotomus</i>). III: New species and records : alterations and additions to the keys	322
GORDON, R. M. ; and CREWE, W. The mechanisms by which mosquitoes and tsetse-flies obtain their blood-meal, the histology of the lesions produced, and the subsequent reactions of the mammalian host ; together with some observations on the feeding of <i>Chrysops</i> and <i>Cimex</i>	334
FRIEDHEIM, E. A. H. Melarsen oxide in the treatment of human trypanosomiasis	357
GORDON, R. M. ; CHWATT, L. J. ; and JONES, C. M. The results of a preliminary entomological survey of loiasis at Kumba, British Cameroons, together with a description of the breeding-places of the vector and suggestions for future research and possible methods of control	364
KERSHAW, W. E. Observations on <i>Litomosoides carinii</i> (Travassos, 1919) Chandler, 1931. I: The development of the first-stage larva ; with a statistical analysis by R. L. Plackett	377
INDEX	i

11

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Thilo Mann-Bahr

THE ACTION *IN VITRO* OF SOME AROMATIC DIAMIDINES ON A SUDAN STRAIN OF *LEISHMANIA INFANTUM*

BY

S. ADLER,

I. TCHERNOMORETZ

AND

M. BER

(From the Department of Parasitology, Hebrew University, Jerusalem)

(Received for publication October 11th, 1947)

Cases of Indian kala-azar respond more readily both to antimony compounds and to aromatic diamidines than do those of Sudanese or Mediterranean origin. Adams and Yorke (1939, 1940) successfully treated Indian kala-azar by eight injections of 1 mgm. per kgm. body-weight stilbamidine. Adler and Rachmilewitz (1939) and Süsskind and Roth (1943) found considerably more intensive treatment necessary in Mediterranean kala-azar. In Syrian hamsters infections of *Leishmania infantum* are more resistant to aromatic diamidines than those of *L. donovani* (Adler and Tchernomoretz, 1941). Kirk and Sati (1940) in the Sudan treated their cases with more than four times the amount of drug employed by Adams and Yorke. In Kirk and Sati's series of cases relapses occurred after 18 injections of 1.2 mgm. per kgm. body-weight.

The Sudan visceral leishmania is generally named *L. donovani*, but since it resembles the Mediterranean form in its relative resistance to aromatic diamidines and antimonials we think that it should be included in *L. infantum*. The action of a number of aromatic diamidines on cultures of an Indian strain of *L. donovani* has been recorded in a previous paper (Adler, Tchernomoretz and Ber, 1945). We decided to compare the *in vitro* action of some of these compounds on cultures of a strain of *L. infantum* from the Sudan, kindly presented by Dr. R. Kirk, of the Stack Memorial Research Laboratories, Khartoum.

The experiments were carried out with the same technique as that previously reported (Adler, Tchernomoretz and Ber, 1945) in the case of *L. donovani*, i.e., 2×10^6 flagellates were inoculated into tubes containing various concentrations of drugs in 5 c.cm. Locke-serum-agar; three series of tubes were used, one incubated at 24° C. for the whole duration of the experiment, one kept at 37° C. for 24 hours, and one at 37° C. for 48 hours, before being transferred to 24° C. The number of flagellates per c.cm. of medium was eventually determined in each tube and compared with controls. Parallel experiments were carried out with L.D. bodies. Suspensions of L.D. bodies were obtained by a method described in a previous paper (Adler and Ashbel, 1940) and were estimated by mixing with a known suspension of fowl erythrocytes and counting the number of L.D. bodies against a number of erythrocytes on stained slides. As in the case of flagellates, 2×10^6 L.D. bodies were inoculated into each tube. Penicillin (5 units per c.cm. of medium) was also added to ensure sterility.*

* During the recent war, together with Colonel J. R. V. Pulvertaft we examined samples of penicillin for their action on leishmania and *Trypanosoma cruzi*. Crude penicillin inhibits the growth of both flagellates, but pure penicillin is without effect.

The compounds tested were stilbamidine, propamidine, pentamidine.

The action of stilbamidine on the Sudan strain of *L. infantum* did not differ significantly, either quantitatively or qualitatively, from that previously recorded in the case of *L. donovani* (Adler, Tchernomoretz and Ber, 1945). There was nothing in the *in vitro* tests to indicate the relatively large difference between the quantity of drug required therapeutically to treat cases of Indian and of Sudan kala-azar respectively.

L. infantum was considerably less sensitive than *L. donovani* to the other two compounds, and the differences noted were well beyond the range of experimental error, e.g., in 1:750,000 pentamidine only a few flagellates of the Indian strain of *L. donovani* survive, and these do not multiply, whereas the Sudan strain grows well and multiplies as much as 50 per cent. when compared with cultures grown in tubes containing no drug. These results are shown in Table I. As in the case of *L. donovani*, both compounds are more effective *in vitro* against the Sudan strain than is stilbamidine.

TABLE I

In vitro action of diamidines on an Indian strain of *L. donovani* and on a Sudan strain of *L. infantum*

Dilution	Propamidine						Pentamidine					
	A	A ₁	B	B ₁	C	C ₁	A	A ₁	B	B ₁	C	C ₁
50,000	—	+	—	—	—	—	—	—	—	—	—	—
100,000	+	+	+	—	—	—	—	—	—	—	—	—
200,000	+	20	+	+	+	+	—	—	—	—	—	—
300,000	+	30	+	+	+	+	—	—	—	—	—	—
400,000	+	50	+	+	+	+	—	+	—	—	—	—
500,000	10	50	10	20	5	5	—	40	—	—	—	—
600,000	10	70	10	40	5	10	—	30	—	—	—	—
750,000	15	70	15	70	15	15	+	50	+	+	+	+
1,000,000	20	80	—	70	—	25	+	50	+	+	+	+
2,000,000	20	80	30	90	30	60	+	100	+	50	+	60
2,500,000	20	100	30	100	—	80	25	—	30	70	40	80
3,000,000	25	—	40	—	40	80	30	—	40	70	55	80
4,000,000	30	—	40	—	50	80	50	—	40	100	50	100
5,000,000	30	—	50	—	50	100	100	—	50	—	60	—
6,000,000	100	—	60	—	70	—	—	—	50	—	70	—
8,000,000	—	—	70	—	70	—	—	—	70	—	80	—
10,000,000	—	—	100	—	100	—	—	—	70	—	80	—
15,000,000	—	—	—	—	—	—	—	—	100	—	100	—

The figures in columns 2-7 are percentages of active flagellates, in relation to the numbers in corresponding control tubes which contained no drug (Adler, Tchernomoretz and Ber, 1945).

A, B, C = An Indian strain of *L. donovani* incubated at 24° C. during the whole experiment, at 37° C. for 24 hours, and at 37° C. for 48 hours respectively.

A₁, B₁, C₁ = *L. infantum* under the same conditions.

+ = Motile flagellates too few to count.

— = No motile flagellates.

ACTION ON LEISHMAN-DONOVAN BODIES

The leishmanicidal titre, as defined by Collier and Lourie (1946), i.e., the lowest concentration at which parasites are not to be found, is definitely lower for L.D. bodies than for flagellates. An equally convenient index is the highest concentration in which a few flagellates survive without multiplying after a standard inoculum of 2×10^6 organisms.

It is obvious that L.D. bodies are more sensitive than flagellates. Beyond the leishmanicidal titre the rate of growth of cultures made from L.D. bodies was not significantly different from those made from flagellates. With all three drugs there are, as previously recorded in the case of *L. donovani*, concentrations which are not directly lethal, which permit motility and survival for 7-10 days, but which inhibit multiplication. When inoculated into a medium free from the drugs these flagellates usually, but not invariably, produce cultures.

TABLE II
Showing relative susceptibility of L.D. bodies and flagellates to various diamidines

Temperature	L.D. bodies			Flagellates		
	1	2	3	1	2	3
At 24° C.	40,000	300,000	2,500,000	10,000	50,000	400,000
At 37° C. for 24 hours and subsequently at 24° C.	100,000	500,000	2,500,000	20,000	100,000	750,000
At 37° C. for 48 hours and subsequently at 24° C.	200,000	600,000	2,500,000	40,000	200,000	750,000

The figures indicate the highest concentration in which very few flagellates are found (without multiplication).

1 = Stilbamidine.

2 = Propamidine.

3 = Pentamidine.

A point of interest in the action of the above three aromatic diamidines on L.D. bodies is that, in appropriate concentrations, flagellates develop although multiplication is inhibited, e.g. :

19.8.47. 2×10^6 circ. L.D. bodies sown in medium containing 1 : 300,000 stilbamidine, and tube incubated for 24 hours at 37° C. and subsequently at 24° C.

29.8.47. Very few flagellates found. Subculture on normal medium positive.

7.9.47. Tube negative.

19.8.47. 2×10^6 circ. L.D. bodies sown in medium containing 1 : 2.5×10^6 pentamidine.

29.8.47. Very few flagellates found. Subculture on normal medium positive.

7.9.47. Tube negative.

REMARKS

In carrying out *in vitro* chemotherapeutic tests on leishmania it is important to use a standard medium and a standard inoculum. Differences in quantitative results between various workers may be due, as Collier and Lourie (1946) have remarked, to differences in the media employed. These authors, using a different medium and a smaller inoculum, obtained much higher effective titres for *L. donovani* for the above compounds than those recorded by us with Locke-serum-agar. In the case of tartar emetic they recorded a leishmanicidal titre of 1 : 40,000 in a fluid medium composed of one part rabbit serum and two parts of a 12.5 per cent. rabbit red-cell solution, whereas we found that the organisms are not destroyed by 1 : 10,000 tartar emetic in 10 per cent. rabbit serum in Locke's solution. It is obviously difficult to compare results obtained by different methods, and we have therefore confined ourselves to a uniform technique for comparative studies on different strains.

SUMMARY

The action *in vitro* of stilbamidine, propamidine and pentamidine was studied on a Sudan strain of *Leishmania infantum* (both L.D. bodies and flagellates).

The lowest concentrations of the three compounds which inhibit multiplication do not interfere with the development of an L.D. body into a flagellate.

Stilbamidine is less effective than the other two drugs on both the L.D. body and flagellate stage *in vitro*.

L.D. bodies were found to be more sensitive than flagellates to the action of the three compounds tested.

Propamidine and pentamidine were found to be less effective on a Sudan strain of *L. infantum* than on an Indian strain of *L. donovani*.

There was no significant quantitative difference between the action of stilbamidine on flagellates of an Indian strain of *L. donovani* and of a Sudan strain of *L. infantum*, although Indian kala-azar responds more readily than Sudan kala-azar to treatment with this drug.

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THE GROWTH OF *ANOPHELES SERGENTI* THEOBALD (DIPTERA, CULICIDAE), WITH SPECIAL REFERENCE TO THE GROWTH OF THE ANAL PAPILLAE IN VARYING SALINITIES

BY

D. S. KETTLE, CAPTAIN, R.A.M.C.

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I. INTRODUCTION

In the course of a malaria survey in the Jordan Valley it was noticed that larvae of *Anopheles multicolor* could be separated from those of *An. sergenti** and *An. superpictus* by the length of the anal papillae. The larvae of *An. multicolor* had small rounded anal papillae which were almost as broad as long, whilst in the other two species the anal papillae were many times longer than they were broad. Later, in the Nile Delta, where there are only four common species of anopheline mosquito, it was found that *An. multicolor* and *An. sergenti* larvae could be separated from the larvae of *An. coustani* and *An. pharoensis* by the length of their anal papillae. In these last two species the anal papillae are lanceolate, like those of *An. sergenti*, but they are proportionately very much longer. This difference is accentuated by the larger size to which the larvae of *An. coustani* and *An. pharoensis* grow, and was found to hold good for larvae collected from a number of natural breeding-places of different salinities.

This use of the length of the anal papillae as a character in the identification of mosquito larvae is not new. Kirkpatrick (1925) used it to separate *Aedes caspius* from *Ae. detritus* and to separate his two variants of *Ae. caspius*, whilst Edwards (1921) and Marshall (1938) repeatedly used the length of the dorsal anal papillae in their keys to larvae of the genus *Aedes*. Yet it has long been recognized that the anal papillae of mosquito larvae reared in salt water are considerably smaller than those reared in fresh water (Martini, 1922).

Gibbins (1932) illustrated differences between the anal papillae of four species of mosquito larvae which he found in both salt and fresh water, but he did not record the salinities of the waters. Jobling (1938) investigated in more detail this relationship between the salinity of the water in which larvae were reared and the length of the anal papillae for the two subspecies of *Culex pipiens*, *C. pipiens pipiens* and *C.p. autogenicus* (= *C. molestus*). He found that in both subspecies the lengths of the anal papillae steadily decreased with increasing salinity. Wigglesworth (1938a) confirmed this for *C.p. autogenicus*, but in *Ae. aegypti* he found that the anal papillae were very long when reared in distilled water and much shorter in 0.1 per cent. NaCl, though there was very little difference in the lengths of the anal papillae of larvae reared in 0.1–0.75 per cent. NaCl solution. Beadle (1939) kept fourth stage larvae of the saline-water mosquito *Ae. detritus* in distilled water and in salinities up to 7.0 per cent. NaCl, but noticed no difference in the length of the dorsal anal papillae, which remained small and rounded. The final

* Throughout this paper the generic names *Anopheles* and *Aedes* will be abbreviated to *An.* and *Ae.* respectively to avoid confusion.

stage of atrophy of the anal papillae of salt-water-breeding mosquito larvae is shown by *Ae. concolor*, in which the anal papillae are said to be absent (Woodhill, 1938).

The value of a character for the identification of species depends upon its constancy, and it was therefore decided to investigate in the laboratory the effect of increasing salinity on the length of the dorsal anal papillae of the four common Egyptian anopheline mosquitoes, and to correlate this change with the range of salinity in which the larvae are found to occur naturally. Unfortunately, the author left Egypt when this effect had been studied in *An. sergenti* only, and comparative data are therefore lacking.

The length of the dorsal anal papillae may be influenced by growth, and the importance of the growth-rate of an organ, when its size is used as a specific character,

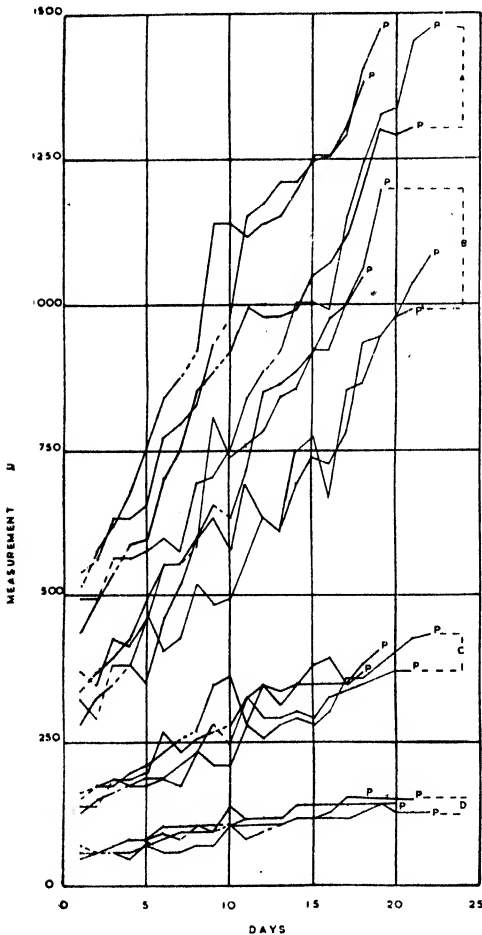


FIG. 1. Growth of four wild caught second stage larvae of *An. multicolor* until pupation (P). The dotted lines denote the days on which ecdysis took place as indicated by the presence of a cast skin. Curves A, B, C and D represent the growth of the thorax breadth, thorax length, length of second abdominal segment and length of dorsal anal papillae respectively.

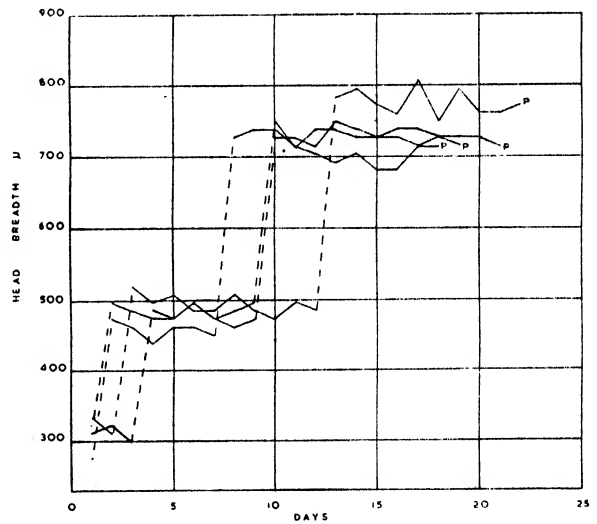


FIG. 2. Growth of head breadth of four wild caught second stage larvae of *An. multicolor* until pupation (P). The dotted lines denote the days on which ecdysis took place as indicated by the presence of a cast skin.

has been stressed by Huxley (1932). It is not sufficient to replace the absolute size of an organ by its size relative to another organ or part of the body, since it is frequently found that the two organs are growing at different rates.

Experiments were therefore begun to provide information on the following problems : (i) Do the anal papillae grow during an instar, or are they constant ? (ii) If they grow during an instar, is their growth-rate the same as that of any other part of the body ? That is, can the absolute size of the anal papillae be replaced by an index which will be independent of growth ?

II. PRELIMINARY EXPERIMENTS WITH *AN. MULTICOLOR*

Preliminary experiments were conducted with second stage larvae of *An. multicolor* which had been collected in the field. They were reared separately in glass capsules and measured daily. (The measurements are defined later.) The growth of four of these larvae is illustrated in figs. 1 and 2, where it will be noticed that the thorax grows more or less steadily in length and breadth throughout an instar and shows no sudden increase at ecdysis (fig. 1), whereas the head breadth remains more or less constant throughout an instar and shows a relatively enormous increase immediately after ecdysis (fig. 2). The second abdominal segment and the dorsal anal papillae also grow steadily in length throughout the whole of the larval life. It has not been possible to insert the measurements of the breadth of the second abdominal segment and the length of the head (figs. 1 and 2) because they are almost the same as those for the thorax length and head breadth respectively.

These experiments indicate that it is possible to separate the different instars of *An. multicolor* on the head breadth. This fact has been noticed in *An. superpictus* and *An. maculipennis* by Bates (1939), who states that 'the larval instar can be determined quite exactly by noting the size of the head, which is quite constant for each species at each stage of growth.'

On account of the high larval mortality due to repeated measuring, it was decided to measure a large number of larvae of varying sizes in each of the four instars. Each larva was to be measured once and then discarded. In this manner the general lines of growth of the various parts of the body and their normal variations would be obtained, whilst individual abnormalities would be minimized.

Since gravid females of *An. multicolor* were rare and those of *An. sergenti* were plentiful in our routine adult collections, the latter species was used in the experiments described in section V.

III. TECHNIQUE

Gravid female *An. sergenti*, obtained from the native villages of Abu Gamus and Abu Sultan in the Suez Canal zone, were placed in small cages of mosquito-netting (6 in. by 6 in. by 6 in.) containing a bowl of tap-water. Eggs, which the females readily laid on the tap-water during the night, were transferred to diluted sea-water equivalent to a 0.25 per cent. solution of sodium chloride within 18 hours of deposition. Between 50 and 100 eggs were placed in a 4 in. enamel bowl containing 100 ml. of solution. When the eggs hatched the larvae were fed on a suspension of baker's yeast, as suggested by Boyd (1926). This proved an ideal food, since first stage larvae were never killed by the surface film as they had been when fed on bread-crumbs in previous experiments. If

more yeast were added to the water than the larvae could eat the water rapidly became foul and fungal growths appeared on the sides of the bowl. Whenever this occurred the water was changed at once.

In order to prevent evaporation from the bowls they were kept in the shade and covered with a glass lid. The water-level was marked with a wax pencil, and distilled water was added on the rare occasions when the water-level dropped.

Throughout these experiments the sea-water used was obtained from Lake Timsah on the Suez Canal and had the following composition :

Sodium chloride	3.56	per cent.
Calcium carbonate	0.02	"
Calcium sulphate	0.17	"
Magnesium chloride	0.43	"
Magnesium sulphate	0.276	"
Unknown	0.224	"
<hr/>		
Total solids	4.680	"

This solution has a salinity of 4.09 per cent. NaCl when estimated as chloride and expressed as the sodium salt, whilst its osmotic pressure, assuming that all the salts are completely ionized, is equivalent to a 4.31 per cent. solution of NaCl. The experimental solutions were made by diluting this sea-water with water from the sweet-water canal and not with distilled water, since the canal water had only a negligible salinity (0.006 per cent. NaCl) and contained organisms which were valuable as larval food. It will be referred to throughout this paper as tap-water. The strengths of the experimental solutions have been based on their chloride contents and not on the total solids or the osmotic pressures, i.e., a solution stated to be equivalent to 0.25 per cent. NaCl will have a total solid content of 0.286 per cent. and an osmotic pressure equivalent to a solution of 0.264 per cent. NaCl. The chloride content of all solutions in which larvae were reared was estimated before and after use or whenever the water was changed, but it was neither practical nor particularly useful to carry out a more detailed analysis. It must be stressed that in no case was sodium chloride used alone as the source of salinity.

When a larva was measured it was placed alive on a slide in a drop of water, and gently covered with a cover-glass, which usually held it by the head capsule, but late fourth stage larvae were secured by their thoraces. If a larva showed any signs of compression or contraction it was rejected—contraction of the abdomen was not uncommon. Each larva was measured once only and then discarded. The measurements were usually made under the 2/3 objective with an eyepiece micrometer in the $\times 6$ ocular, except that any measurement of less than 10 small divisions on the micrometer, i.e., 115μ , was made under the 1/6 objective. The following seven measurements were made on each larva :

- (i) *Head breadth*—the widest part of the head capsule.
- (ii) *Head length*—the distance from the anterior edge of the fronto-clypeal plate between the inner clypeal hairs to the hind edge of the collar.
- (iii) *Thorax breadth*—the widest portion of the mesothorax.
- (iv) *Thorax length*—the distance from the anterior margin of the prothorax between the inner submedian prothoracic hairs and the middle of the first tergal plate. This includes morphologically part of the first abdominal segment, but the tergal plate formed a useful

* I am indebted to Major Holbrooke, R.A.M.C., for this analysis.

landmark. When the tergal plates were not developed the abdomen appeared banded by narrow transverse light areas in which they would later develop. Therefore, when the tergal plates were absent the centre of the first light band was taken as the posterior limit of the thorax.

(v) *Breadth of the second abdominal segment*—the widest part of the second abdominal segment between the bases of the lateral balancer hairs, nos. 6 and 7.

(vi) *Length of the second abdominal segment*—the distance from the centre of the second to the centre of the third tergal plate (although morphologically this measurement includes part of the third abdominal segment and excludes part of the second). When the tergal plates were not developed the measurement was taken from the centre of the second to the centre of the third light transverse band.

(vii) *Length of the dorsal anal papillae*—measured from their insertion into the anal membrane to their tips; in the rare instances when the papillae were of different lengths the mean was noted.

IV. METHOD OF ANALYSIS

It would have been preferable to have plotted the measurements of the different regions of the body against the total length of the larva, but this measurement was difficult to obtain since the larva was usually several times larger than the field of the microscope and its abdomen was frequently flexed. As the largest of the seven recorded measurements was the thorax breadth, it was taken as indicative of the size of the larva and all the other measurements were plotted against it.

Huxley (1924) has shown that the growth of an organ can be represented by the equation

$$y = bx^k, \quad 1$$

where y = the magnitude of the organ whose growth is being measured, x = the magnitude of the animal as measured by some standard linear measurement (in this paper x = the thorax breadth), and b and k are constants. Of these constants b is of no biological significance, whereas k is of great importance since it represents the growth coefficient of the organ relative to the growth of the body. If k has a value of unity, then the organ is growing at the same rate as the body, and its growth may be said to be isogonic with that of the body; if k has a value greater than unity, the organ shows positive heterogony; if k be less than unity, it shows negative heterogony.

Formula 1 can be rewritten thus (Huxley, 1924):

$$\log y = \log b + k \log x, \quad 2$$

and this form will be used throughout the present paper, since it shows that there is a linear relationship between $\log y$ and $\log x$.

To obtain the growth coefficients of the measurements defined in section III, 500 larvae of *An. sergenti*, reared in diluted sea-water equivalent to a 0.25 per cent. NaCl solution, were measured. Of these, 125 were in each of the four larval instars, the instar being determined from the breadth of the head capsule. The larvae within each instar were divided into six classes on the size of the thorax breadth, and the mean measurements of the different regions of the body for each class were determined to the nearest 0.1μ , except for values greater than $1,000\mu$ which were calculated to the nearest 1μ . The class means for each measurement were plotted on a logarithmic grid, as, for example, in fig. 3.

In order to reduce the data to an expression of the type mentioned above, the class means were converted to five-figure logarithms, and the coefficient was calculated for the regression of the logarithm of each region of the body ($= \log y$) on the logarithm of the thorax breadth ($= \log x$).^{*} The complete expression is obtained by substituting the calculated regression coefficient in the formula

$$\log y = a + d (\log x - c), \quad \dots \dots \dots 3$$

where x = thorax breadth, y = region of the body, a = mean of values of $\log y$, c = mean of values of $\log x$, and d = regression coefficient of $\log y$ on $\log x$. This gives the best straight line to fit the data. It will be noticed that the constant k in Huxley's formula is identical with the regression coefficient of $\log y$ on $\log x$, and this statistic and its standard error can be calculated accurately. Whilst it is not very important biologically to determine the growth coefficient of an organ with great accuracy, it is of importance to know whether a difference observed between growth coefficients of various organs is, on the data available, significant. In the following sections the regression coefficients and their standard errors have been calculated by the method detailed in Fisher (1944) para. 26, and comparisons between regression coefficients have been made by the method given in para. 26, 1.

The calculation of the expression giving the growth of a region of the body gives no indication as to the deviation shown by individual larvae from this general line. This can best be represented by a distribution diagram in which the observations are grouped around mean values so that the range of each group is approximately ± 5 per cent. of the mean value, and the number of examples in each group is then entered on a logarithmic grid, as in fig. 4.

V. THE GROWTH OF *AN. SERGENTI* LARVAE IN DILUTED SEA-WATER EQUIVALENT TO A 0.25 PER CENT. SOLUTION OF NaCl

When the data were analysed it was discovered that there were three different growth patterns: the thorax and second abdominal segment grew steadily throughout larval life; the head showed hardly any increase in size during each instar but increased enormously at each ecdysis; and the anal papillae grew by a combination of both patterns—that is, they lengthened considerably during an instar and also increased suddenly at ecdysis.

(a) *Growth of the Thorax and Abdomen*

The 24 mean class measurements of the thorax are plotted on a logarithmic grid in fig. 3. They show that the thorax grows steadily throughout larval life, since there is no appreciable difference in the thorax length of larvae of similar size (thorax breadth) but belonging to different instars (fig. 3, Table I). The expression which has been calculated to fit these observations is:

$$\log \text{thorax length} = 1.09399 \log \text{thorax breadth} - \log 2.5522, \quad \dots \dots \dots 4$$

and the growth coefficient is 1.09399 ± 0.00630 .

Gray (1929) has pointed out that, when logarithmic co-ordinates are used, graphic methods are inadequate to test the agreement between an equation and the data. Therefore

^{*} No weighting coefficient was used to allow for the different number of examples in each class.

the observed values of the thorax length have been compared with values calculated by substituting the observed thorax breadth into formula 4 (see Table I).

The mean percentage difference between the observed and the calculated values is the mean of the percentage differences, summed irrespective of their sign (Table I). This is 1.58 per cent. and indicates a close agreement between the derived expression and the observations. The mean percentage difference as defined here is quite distinct from the 'mean deviation' calculation of Huxley (1932), in which the percentage differences

TABLE I
Growth of the thorax of *An. sergenti* reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl

(a) Instar no.	(b) Class no.	(c) No. of larvae	(d) Mean thorax breadth in μ	(e) Mean observed thorax length in μ	(f) Calculated thorax length in μ	(g) Difference in μ	(h) Percentage difference
1	i	8	190	122	122	0	0
	ii	44	213	137	138	- 1	-0.7
	iii	42	235	152	154	- 2	-1.3
	iv	20	258	173	170	+ 3	+1.8
	v	7	285	192	190	+ 2	+1.1
	vi	4	303	205	203	+ 2	+1.0
2	i	12	287	197	191	+ 6	+3.1
	ii	27	334	230	226	+ 4	+1.8
	iii	41	372	255	254	+ 1	+0.4
	iv	24	412	287	285	+ 2	+0.7
	v	14	462	320	322	- 2	-0.6
	vi	7	508	357	358	- 1	-0.3
3	i	12	482	325	337	-12	-3.6
	ii	29	528	363	373	-10	-2.7
	iii	34	586	411	418	- 7	-1.7
	iv	22	645	449	464	-15	-3.2
	v	19	697	501	505	- 4	-0.8
	vi	9	771	555	564	- 9	-1.6
4	i	20	679	488	491	- 3	-0.6
	ii	27	760	546	556	-10	-1.8
	iii	29	861	647	636	+11	+1.7
	iv	31	954	734	712	+22	+3.1
	v	13	1,037	806	780	+26	+3.3
	vi	5	1,150	882	874	+ 8	+0.9

Mean percentage difference = 1.58

The calculated values for the thorax length (column f) have been obtained by substituting the values for the thorax breadth (column d) in the expression :

$$\log \text{thorax length} = 1.09399 \log \text{thorax breadth} - \log 2.5522.$$

are summed algebraically. Thus the 'mean deviation' of Huxley is not a measure of the deviation between the observed and the calculated values, but can be more aptly described as a test of the accuracy of the calculated formula. This is clearly shown in Tables I, II and III, where the mean percentage differences are 1.58, 2.27 and 3.21, whilst the 'mean deviations' are 0.00 per cent., 0.04 per cent. and 0.06 per cent. respectively.

The distribution of the 500 separate measurements of the thorax length is shown in fig. 4, and the variation from the calculated line is consistent throughout larval life.

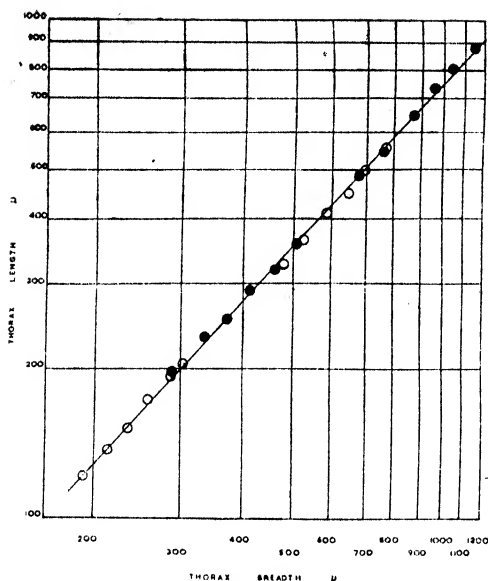


FIG. 3. Growth of thorax of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Open circles represent first and third instars; closed circles represent second and fourth instars. Data from Table I. Straight line fitted according to formula 4 in text. Logarithmic co-ordinates.

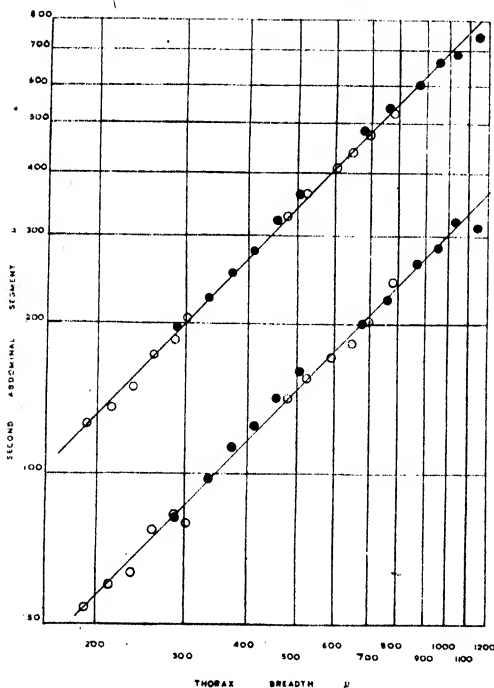


FIG. 5. Growth of second abdominal segment of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Upper and lower straight fitted lines represent breadth (formula 5) and length (formula 6) respectively of second abdominal segment. Data from Tables II and III. Open and closed circles as in fig. 3. Logarithmic co-ordinates.

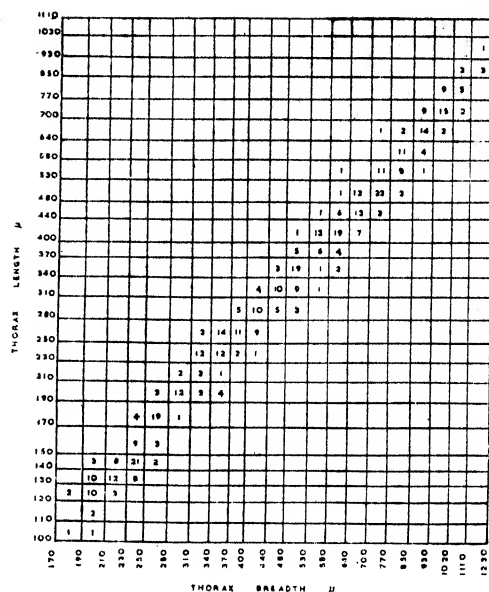


FIG. 4. Distribution of individual measurements of thorax length of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Logarithmic co-ordinates.

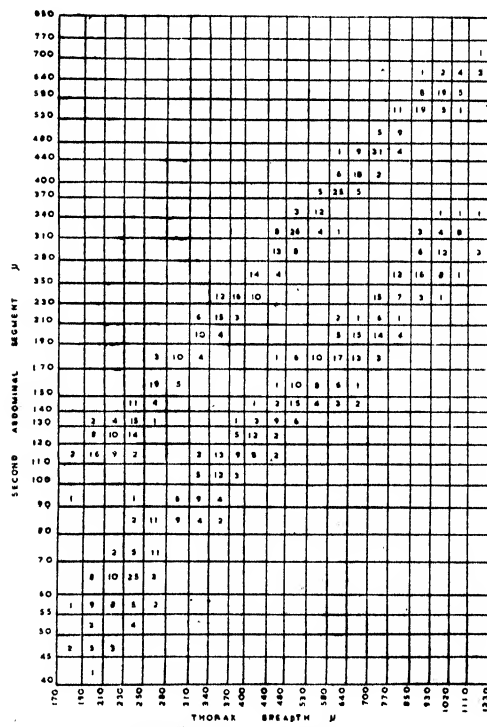


FIG. 6. Distribution of individual measurements of second abdominal segment of *An. sergenti* larvae reared in sea-water equivalent to 0.25 per cent. NaCl solution. Upper and lower figures represent breadth and length respectively. Logarithmic co-ordinates.

The biological importance of the growth coefficient depends on whether it differs significantly from unity. The probability (P) that this value could have been obtained from a population in which the true growth coefficient was unity is very much less than one chance in a hundred ($P < 0.01$). Indeed, for P to have a value of less than 0.01 the statistic t should be greater than 2.819, and in this instance $t = 14.92$. Therefore, the thorax grows heterogonically, since it grows more rapidly in length than it does in breadth.

TABLE II

Growth of the second abdominal segment of *An. sergenti* reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl

(a) Instar no.	(b) Class no.	(c) No. of larvae	(d) Mean thorax breadth in μ	(e) Mean observed abdominal length in μ	(f) Calculated abdominal length in μ	(g) Difference in μ	(h) Percentage difference
1	i	8	190	54	54	0	0
	ii	44	213	59	61	- 2	-3.3
	iii	42	235	64	67	- 3	-4.5
	iv	20	258	77	74	+ 3	+4.1
	v	7	285	83	82	+ 1	+1.2
	vi	4	303	79	87	- 8	-9.2
2	i	12	287	82	83	- 1	-1.2
	ii	27	334	98	97	+ 1	+1.0
	iii	41	372	113	108	+ 5	+4.6
	iv	24	412	124	120	+ 4	+3.3
	v	14	462	142	135	+ 7	+5.2
	vi	7	508	160	149	+11	+7.4
3	i	12	482	142	141	+ 1	+0.7
	ii	29	528	154	155	- 1	-0.6
	iii	34	586	170	172	- 2	-1.2
	iv	22	645	183	190	- 7	-3.7
	v	19	697	202	206	- 4	-1.9
	vi	9	771	242	228	+14	+6.1
4	i	20	679	200	200	0	0
	ii	27	760	222	225	- 3	-1.3
	iii	29	861	263	256	+ 7	+2.7
	iv	31	954	282	285	- 3	-1.1
	v	13	1,037	319	310	+ 9	+2.9
	vi	5	1,150	311	345	-34	-9.9

Mean percentage difference = 3.21

The calculated values for the length of the second abdominal segment (column f) have been obtained by substituting the values for the thorax breadth (column d) in the expression :

$$\log \text{ length second abdominal segment} = 1.02868 \log \text{ thorax breadth} - \log 4.0815.$$

The growth of the abdomen, as shown by that of the second abdominal segment, proceeds steadily, irrespective of ecdyses (fig. 5). The mean class measurements of its length and breadth are given in Tables II and III, and from these the following expressions have been derived :

$$1. \log \text{ length of second abdominal segment} = 1.02868 \log \text{ thorax breadth} - \log 4.0815, \quad \dots \quad 5$$

$$\text{Growth coefficient} = 1.02868 \pm 0.01708$$

2. $\log \text{ breadth of second abdominal segment} = 1.03314 \log \text{ thorax breadth} - \log 1.8103$, 6
 Growth coefficient $= 1.03314 \pm 0.01116$

The growth coefficient of the breadth of the second abdominal segment shows a highly significant difference from unity ($P < 0.01$), whilst that of the length of the second abdominal segment does not significantly differ from 1.0 (P lies between 0.1 and 0.2). The

TABLE III

Growth of the second abdominal segment of *An. sergenti* reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl

(a) Instar no.	(b) Class no.	(c) No. of larvae	(d) Mean thorax breadth in μ	(e) Mean observed abdominal breadth in μ	(f) Calculated abdominal breadth in μ	(g) Difference in μ	(h) Percentage difference
1	i	8	190	126	125	+ 1	+0.8
	ii	44	213	135	141	- 6	-4.3
	iii	42	235	147	156	- 9	-5.8
	iv	20	258	173	171	+ 2	+1.2
	v	7	285	185	190	- 5	-2.6
	vi	4	303	205	202	+ 3	+1.5
2	i	12	287	194	191	+ 3	+1.6
	ii	27	334	223	224	- 1	-0.4
	iii	41	372	252	250	+ 2	+0.8
	vi	24	412	281	278	+ 3	+1.1
	v	14	462	320	313	+ 7	+2.2
	vi	7	508	361	345	+16	+4.6
3	i	12	482	327	326	+ 1	+0.3
	ii	29	528	365	359	+ 6	+1.7
	iii	34	586	407	400	+ 7	+1.8
	iv	22	645	439	442	- 3	-0.7
	v	19	697	475	478	- 3	-0.6
	vi	9	771	528	531	- 3	-0.6
4	i	20	679	486	465	+21	+4.5
	ii	27	760	537	523	+14	+2.7
	iii	29	861	612	595	+17	+2.9
	iv	31	954	661	661	0	0
	v	13	1,037	688	721	-33	-4.6
	vi	5	1,150	744	802	-58	-7.2

Mean percentage difference = 2.27

The calculated values for the breadth of the second abdominal segment (column f) have been obtained by substituting the values for the thorax breadth (column d) in the expression:
 $\log \text{ breadth second abdominal segment} = 1.03314 \log \text{ thorax breadth} - \log 1.8103$.

lack of statistical significance in the latter test is undoubtedly due to the greater deviation shown by those measurements. This is clearly shown in figs. 5 and 6 and in Tables II and III, where the mean percentage difference of the length of the second abdominal segment is 3.21, compared with 2.27 for its length and 1.58 for the thorax length. It seems reasonable to assume that if a larger sample had been taken the difference would have been significant, particularly since the growth coefficients of the length and breadth of the second abdominal segment are so similar. It is highly probable, but not completely

proven, that the growth of the second abdominal segment is isogonic in itself but heterogonic when compared with either the breadth or the length of the thorax.

(b) *Growth of the Head*

The distribution of the individual measurements of the head is shown in figs. 7 and 8. Whilst the measurements of the head breadth fall into four isolated groups corre-

TABLE IV
Growth of the head of *An. sergenti* reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl

(a) Instar no.	(b) Class no.	(c) No. of larvae	(d) Mean thorax breadth in μ	(e) Mean observed head length in μ	(f) Mean observed head breadth in μ	(g) Calculated head breadth in μ	(h) Difference in μ	(i) Percentage difference
1	i	8	190	142	162	163	- 1	-0.6
	ii	44	213	142	167	166	+ 1	+0.6
	iii	42	235	149	166	168	- 2	-1.2
	iv	20	258	167	173	170	+ 3	+1.8
	v	7	285	177	174	173	+ 1	+0.6
	vi	4	303	194	173	174	- 1	-0.6
Mean percentage difference = 0.9								
2	i	12	287	218	244	251	- 7	-2.8
	ii	27	334	232	260	256	+ 4	+1.6
	iii	41	372	236	264	259	+ 5	+1.9
	iv	24	412	250	263	262	+ 1	+0.4
	v	14	462	267	268	265	+ 3	+1.1
	vi	7	508	283	262	268	- 6	-2.2
Mean percentage difference = 1.7								
3	i	12	482	354	399	390	+ 9	+2.3
	ii	29	528	347	392	393	- 1	-0.3
	iii	34	586	361	385	396	-11	-2.8
	iv	22	645	376	394	398	- 4	-1.0
	v	19	697	395	400	400	0	0
	vi	9	771	418	411	402	+ 9	+2.2
Mean percentage difference = 1.4								
4	i	20	679	531	578	584	- 6	-1.0
	ii	27	760	546	592	589	+ 3	+0.5
	iii	29	861	553	600	595	+ 5	+0.8
	iv	31	954	564	598	599	- 1	-0.2
	v	13	1,037	573	608	603	+ 5	+0.8
	vi	5	1,150	585	602	608	- 6	-1.0
Mean percentage difference = 0.7								

The calculated values for the head breadth (column g) have been obtained by substituting the values for the thorax breadth (column d) in expressions 7-10 given in the text.

sponding to the four larval instars (fig. 7), there is a slight amount of overlapping between the measurements of the head length in the earlier instars, which is due to the addition of the collar on the posterior border of the head capsule (fig. 8). The collar is absent in newly moulted larvae, but is rapidly laid down throughout the life of all instars, except the fourth, until it forms an appreciable proportion of the head length in larvae which are near ecdysis.

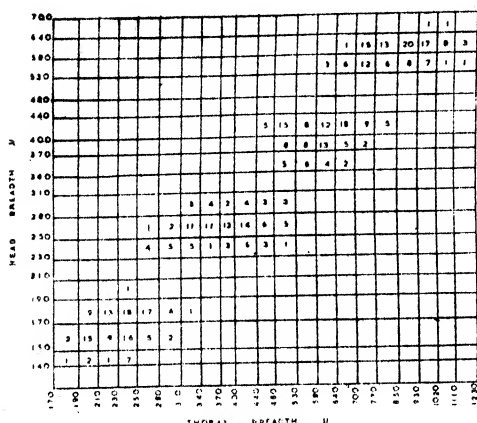


FIG. 7. Distribution of individual measurements of head breadth of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Logarithmic co-ordinates.

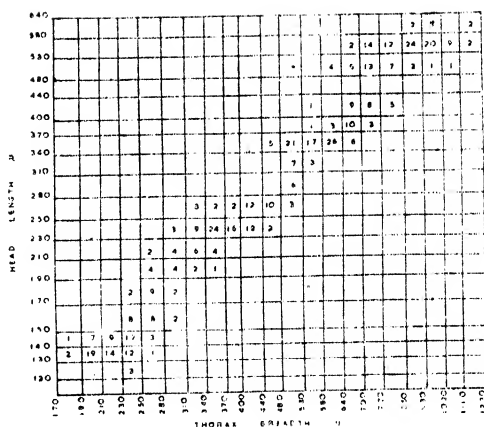


FIG. 8. Distribution of individual measurements of head length of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Logarithmic co-ordinates.

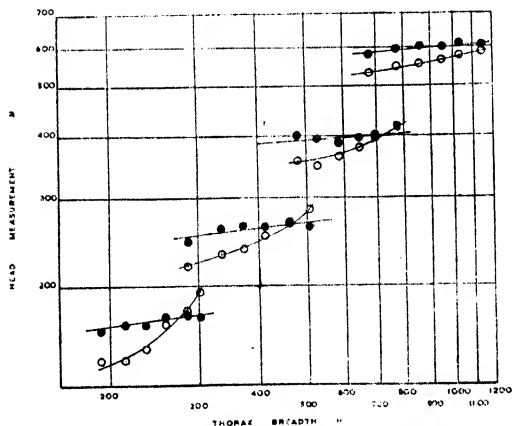


FIG. 9. Growth of head of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Open circles represent head length; closed circles represent head breadth. Straight lines fitted to head breadth readings according to formulae 7-10 in text. Logarithmic co-ordinates.

The mean class measurements of the head are plotted in fig. 9. Since the co-ordinates are logarithmic and the increase in the head length is mainly due to the growth of the collar, it is clear that at its maximum development the collar forms a larger proportion of the head length in the first instar than in the others; indeed the ratio of the collar to the head length steadily decreases in the later instars until in the fourth stage the collar forms a relatively insignificant ring at the hind border of the head. Moreover, the collar is not laid down equally throughout the instar, but is formed more rapidly at the end of an instar than at the beginning.

The mean class measurements of the head breadth show a small, very uneven, increase throughout an instar (fig. 9, Table IV). The following expressions have been calculated from the data:

$$\begin{array}{ll} \text{Instar 1: } \log y = 0.14483 \log x + \log 76.266, & \dots \dots \dots 7 \\ \text{Instar 2: } \log y = 0.11625 \log x + \log 130.02, & \dots \dots \dots 8 \\ \text{Instar 3: } \log y = 0.06866 \log x + \log 255.33, & \dots \dots \dots 9 \\ \text{Instar 4: } \log y = 0.07707 \log x + \log 353.23, & \dots \dots \dots 10 \end{array}$$

where y = head breadth and x = thorax breadth.

The growth coefficients and the probability (P) that they differ significantly from zero are:

$$\begin{array}{llll} \text{Instar 1: } \text{growth coefficient} = 0.14483 \pm 0.0312 & P < 0.01 \\ \text{Instar 2: } \text{,,} \text{,,} = 0.11625 \pm 0.0507 & P = 0.05 - 0.1 \\ \text{Instar 3: } \text{,,} \text{,,} = 0.06866 \pm 0.0540 & P = 0.2 - 0.3 \\ \text{Instar 4: } \text{,,} \text{,,} = 0.07707 \pm 0.0215 & P = 0.02 - 0.05 \end{array}$$

It appears that only in the first instar does the growth coefficient significantly differ from zero, and it is probably best to regard the apparent growth of the head breadth more in the nature of a stretching of the rigid head capsule, brought about by pressure exerted by the increasing head tissues, than as growth of an organ free to expand in all directions. The formulae to fit the curves of the head length have not been calculated.

TABLE V

Two sets of measurements (in μ) made within five minutes of each other on a single first stage larva of *An. sergenti* immediately before and after ecdysis

	Head		Thorax		Second abdominal segment	
	Breadth	Length	Breadth	Length	Breadth	Length
Before ecdysis ...	128	116	265	184	173	Not made
After ecdysis ...	230	219	276	196	184	Not made

It was not possible to measure the lengths of the second abdominal segment and dorsal anal papillae before moulting began.

The fact that no larva was found with a head breadth intermediate between the size characteristic for one instar and the next suggests that the transition is a rapid one. This was strikingly confirmed when on two occasions first stage larvae moulted whilst being measured. The measurements of one of these larvae are given in Table V, and it is apparent that the head shows a dramatic increase in size when the larva moults, whilst the other parts of the body remain more or less constant.

TABLE VI
Head breadth of *An. sergenti* and Dyar's law

Instar no.	Mean observed head breadth in μ	Calculated head breadth in μ	Difference in μ	Percentage difference
1	168	169	-1	-0.6
2	261	258	+3	+1.4
3	394	393	+1	+0.5
4	595	599	-4	-0.7

Mean percentage difference = 0.8

The calculated head breadth has been obtained by substituting the instar number into formula 12.
 $1/r = 1.524$.

It is interesting to speculate on the means by which this sudden increase might be achieved. Wigglesworth (1938b) noticed that larvae of *Ae. aegypti* swallowed water both before and during moulting. He also observed peristaltic waves which advanced the larva through the split in the head away from the cast skin. Similar waves, which seemed to force the body fluids into the head, were noticed by the present author. A possible sequence of events is that in a newly moulted larva the head tissues do not occupy all the available space—that is, the haemocoel may be relatively large. As the head tissues increase, however, they fill the head capsule and continue to grow, so that they set up an internal pressure on the head capsule, which results in the 'stretching' of the head breadth noted earlier, and overflow into a previously non-existent region of the body—the neck, over which the collar is formed. This agrees with the relatively late development of the collar in the duration of an instar and in the small size of the collar in the fourth stage, as in the fourth stage the larval head tissues have reached maximum development and are due to be broken down in the pupa and rebuilt into the adult structures. When the time for moulting is near, the larva swallows water to increase the volume of the body fluids, and then by peristaltic movements of the abdomen forces the body fluids into the head capsule, which splits along a preformed line of weakness—the epicranial suture. The head which emerges rapidly acquires the size characteristic of the new instar, but the neck region disappears, absorbed into the new head.

TABLE VII
Determination of the head breadth at ecdysis by a growth partition coefficient

(a) Stage of larva	(b) Average thorax breadth after ecdysis in μ	(c) Head breadth after ecdysis in μ		(e) Difference in μ	(f) Percentage difference
		Average	Calculated		
After hatching ...	200	164	166	-2	-1.2
" 1st moult ...	295	251	245	+6	+2.5
" 2nd " ...	480	390	398	-8	-2.0
" 3rd " ...	700	585	581	+4	+0.7

Mean percentage difference = 1.6

The average thorax breadth (column b) has been obtained from fig. 7 by inspection. The average head breadth (column c) has been obtained by substituting the values in column b into expressions 7-10, and the calculated values (column d) by substituting column b in formula 13.

Long ago it was shown that the breadth of the head capsule of lepidopterous larvae increases in regular geometrical progression from instar to instar (Dyar, 1890). The ratio of increase (r) was calculated from the expression :

$$r = \frac{a}{b}, \quad \dots \dots \dots 11$$

where a = head breadth of larval instar $x - 1$, and b = head breadth of larval instar x .

Later authors have used the reciprocal of this ratio (e.g., Gaines and Campbell, 1935; Teissier, 1936), and this practice will be followed here. The mean head breadth for each instar has been plotted against the instar number in fig. 10 (see also Table VI). The head breadth of *An. sergenti* increases in regular geometrical progression, so that

$$\log \text{head breadth} = 0.18295 \text{ instar number} + 2.04528, \quad \dots \dots \dots 12$$

and the ratio of increase ($1/r$) is 1.524 ± 0.009 .

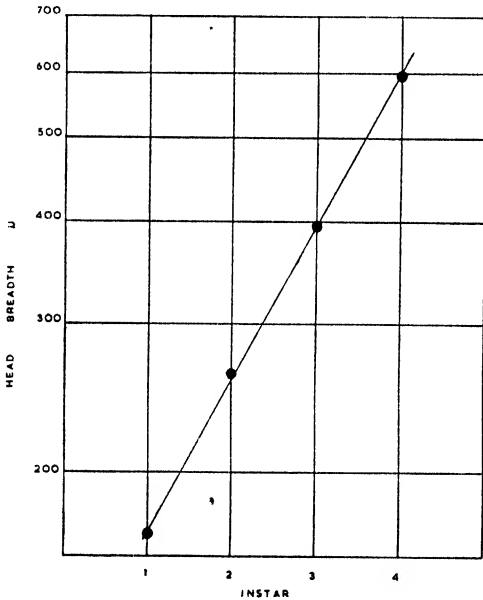


FIG. 10. Dyar's law and the head breadth of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Data from Table VI.

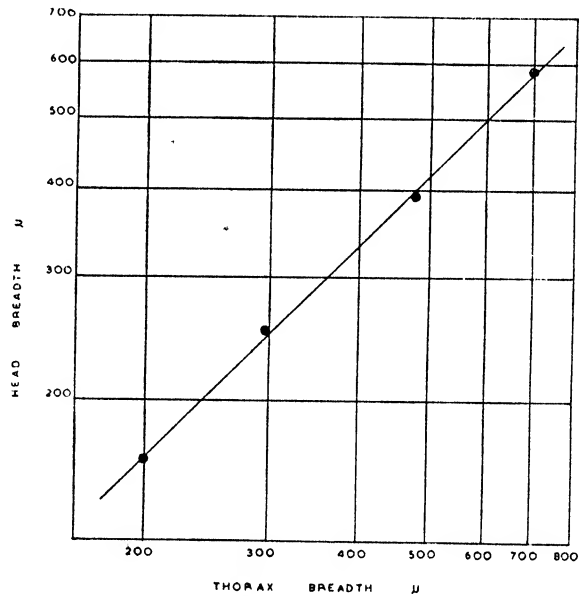


FIG. 11. The determination of the head breadth of *An. sergenti* at ecdysis by a growth partition coefficient between the thorax breadth and the head breadth. Data from Table VII. Logarithmic co-ordinates.

Dyar's law suggests that the size of the head depends upon the ratio of increase, the number of moults and the initial head breadth. Huxley (1932) has suggested that the size of an organ is dependent upon a growth partition coefficient which limits the size of an organ relative to the rest of the body. He points out that the weight of antlers borne by *Cervus elaphus* and *Capreolus capreolus* is dependent upon the weight of the animal on which they occur (Huxley, 1927), and that when an organ capable of complete regeneration is amputated it will be restored under normal favourable conditions to its proportionate size (Huxley, 1932). The growth partition coefficient is a general conception of which the growth coefficient is a special example. Can Dyar's law and the idea of a growth partition coefficient be reconciled?

If the head breadth of a larva is determined at moulting by a growth partition coefficient, and if the larva moults at regular geometrical increases of size, then clearly the head breadth will appear to obey Dyar's law. A similar suggestion has been made by Teissier (1936), although he makes no mention of the growth partition coefficient. Teissier writes: 'La loi de Dyar apparaît ainsi comme une conséquence immédiate de la périodicité régulière des processus physiologiques qui commandent la mue.'

From fig. 7 it is possible to obtain an approximation to the average thorax breadth at the various larval ecdyses. These figures are given in Table VII, and have been substituted into formulae 7-10 given above, in order to obtain the average head breadth at each ecdysis. The two sets of figures are plotted in fig. 11 and approximate to a line of the formula

$$\log \text{ head breadth} = 0.99978 \log \text{ thorax breadth} - 0.08032, \quad \dots \quad 13$$

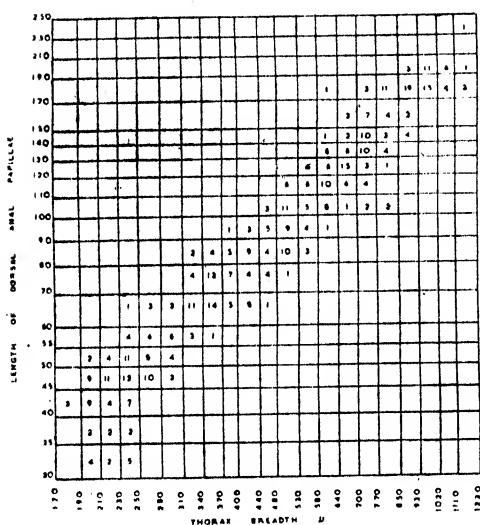


FIG. 12. Distribution of individual measurements of length of dorsal anal papillae of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Logarithmic co-ordinates.

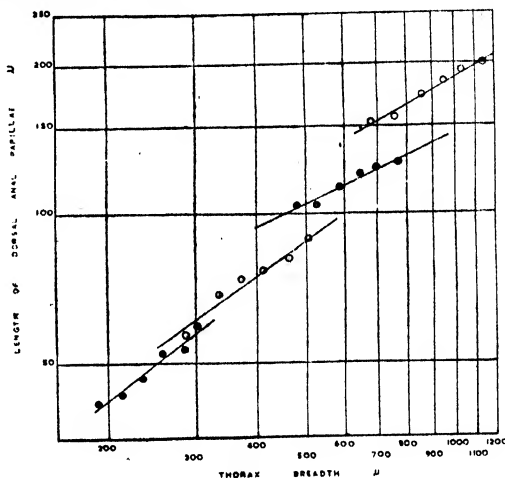


FIG. 13. Growth in length of dorsal anal papillae of *An. sergenti* larvae reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl. Closed circles represent first and third instars; open circles represent second and fourth instars. Straight lines fitted to readings according to formulae 14-17 in text. Logarithmic co-ordinates.

The agreement between the data and this formula is sufficiently close to justify the statement that in *An. sergenti* the head breadth can be considered as being determined by either Dyar's law or a growth partition coefficient. In this case formula 12 shows a smaller percentage deviation from the data than formula 13 (cf. Tables VI-VII); this is due to the difficulty of obtaining accurately the mean thorax breadth at ecdysis. It is likely that the growth partition coefficient is the more general conception, of which Dyar's law is a special example. In this connection it would be interesting to re-examine some of the species which do not appear to obey Dyar's law, and to see whether this be due to the absence of a growth partition coefficient or to ecdysis occurring at irregular intervals. The latter would appear to be the more likely explanation. It therefore appears that the head breadth of *An. sergenti* can be regarded as being determined by a growth partition coefficient between the head and the body; moreover, the 'growth coefficient' for the

head breadth is 0.99978 ± 0.02844 , or the same as that for the thorax breadth. This is unusual, since in most animals the head shows marked negative heterogony.

(c) *Growth of the Dorsal Anal Papillae*

When the individual measurements of the dorsal anal papillae are plotted against the thorax breadth on a logarithmic grid they appear to be scattered about a straight

TABLE VIII

Growth of the dorsal anal papillae of *An. sergenti* reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl

(a) Instar no.	(b) Class no.	(c) No. of larvae	(d) Mean thorax breadth in μ	(e) Mean observed length of dorsal anal papillae in μ	(f) Calculated length of dorsal anal papillae in μ	(g) Difference in μ	(h) Percentage difference
1	i	8	190	42	41	+1	+2.4
	ii	44	213	43	45	-2	-4.4
	iii	42	235	47	48	-1	-2.1
	iv	20	258	53	51	+2	+3.9
	v	7	285	53	55	-2	-3.6
	vi	4	303	59	58	+1	+1.7
Mean percentage difference =							3.0
2	i	12	287	57	60	-3	-5.0
	ii	27	334	69	66	+3	+4.5
	iii	41	372	74	72	+2	+2.8
	iv	24	412	77	77	0	0
	v	14	462	81	83	-2	-2.4
	vi	7	508	89	89	0	0
Mean percentage difference =							2.5
3	i	12	482	104	103	+1	+1.0
	ii	29	528	104	107	-3	-2.8
	iii	34	586	113	112	+1	+0.9
	iv	22	645	120	118	+2	+1.7
	v	19	697	123	122	+1	+0.8
	vi	9	771	126	128	-2	-1.6
Mean percentage difference =							1.5
4	i	20	679	152	150	+2	+1.3
	ii	27	760	156	160	-4	-2.5
	iii	29	861	172	171	+1	+0.6
	iv	31	954	183	181	+2	+1.1
	v	13	1,037	192	190	+2	+1.1
	vi	5	1,150	198	201	-3	-1.5
Mean percentage difference =							1.4

The calculated values for the dorsal anal papillae (column f) have been obtained by substituting the values for the thorax breadth (column d) in expressions 14-17 given in the text.

line in a broad band (fig. 12). This suggests that, although the papillae show great individual variation in length, they grow steadily throughout larval life. However, when the mean class measurements are plotted there is complete isolation of the papilla lengths of the second, third and fourth instars, although there is considerable overlapping in their sizes (thoraces breadth). It is apparent that although there is growth during an instar there is also an appreciable increase in anal papillae length at each ecdysis (fig. 13,

Table VIII). The following straight lines have been fitted to the mean class measurements of each instar :

Instar 1 : $\log y = 0.74791 \log x - \log 1.2401$,	14
Instar 2 : $\log y = 0.70607 \log x + \log 1.0946$,	15
Instar 3 : $\log y = 0.46755 \log x + \log 5.7064$,	16
Instar 4 : $\log y = 0.55421 \log x + \log 4.0445$,	17

where x = thorax breadth and y = length of dorsal anal papillae.

The intra-instar growth coefficients and their standard errors are :

Instar 1 : 0.74791 ± 0.08381

Instar 2 : 0.70607 ± 0.07592

Instar 3 : 0.46755 ± 0.06012

Instar 4 : 0.55421 ± 0.03884

Although the differences between the growth coefficient of the third instar and those of the first two instars are mathematically significant ($P = 0.02 - 0.05$), they do not appear to have any biological importance.

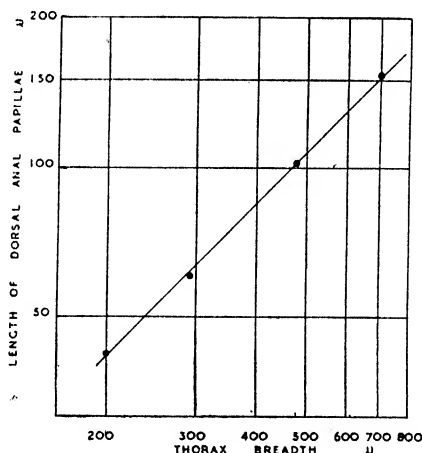


FIG. 14. The determination of the length of the dorsal anal papillae of *An. sergenti*, reared in diluted sea-water equivalent to a solution of 0.25 per cent. NaCl, by a growth partition coefficient between it and the thorax breadth. Data from Table IX. Logarithmic co-ordinates.

It is of interest to consider whether the length of the anal papilla is regulated at ecdysis by a growth partition coefficient. This can be evaluated by the same method as that used for the head breadth (see section (b) above). The average papilla length at the commencement of each instar has been obtained by substituting the mean thorax breadth of newly moulted and newly emerged larvae into formulae 14-17. The four pairs of measurements are then plotted on a logarithmic grid and the best straight line fitted to them (fig. 14, Table IX). This is :

$$\log y = 1.02855 \log x - \log 5.5815, \quad 18$$

where x = thorax breadth and y = dorsal anal papilla length; the mean percentage deviation from this formula is 0.7. Therefore the length of the dorsal anal papillae is

TABLE IX

Determination of the anal papillae length at ecdysis by a growth partition coefficient

(a) Stage of larvae	(b) Average thorax breadth after ecdysis in μ	(c) (d) Dorsal anal papillae length after ecdysis in μ		(e) Difference in μ	(f) Percentage difference
		Average	Calculated		
After hatching ...	200	42	42	0	0
" 1st moult ...	295	61	62	-1	-1.6
" 2nd " ...	480	102	102	0	0
" 3rd " ...	700	153	151	+2	+1.3

Mean percentage difference = 0.7

The average thorax breadth (column b) has been obtained from fig. 7 by inspection. The average dorsal anal papillae length (column c) has been obtained by substituting the values in column b into expressions 14-17. The calculated values (column d) have been obtained by substituting the values in column b in expression 18.

determined at ecdysis by a constant growth ratio. It is also of note that the over-all growth coefficient for the whole of larval life is 1.02855 ± 0.02558 ; moreover, this approximates to the growth coefficients of the length and breadth of the second abdominal segment, which were 1.02868 and 1.03314 respectively, and suggests that the growth coefficient for the abdomen as a whole is about 1.03.

The growth of the anal papillae consists of a steady lengthening during an instar and a sudden increase at ecdysis, but the total amount of growth achieved is regulated by a growth partition coefficient. It is not clear why the growth of the anal papillae should follow this curious pattern, since, unlike the head, they are not confined by a rigid exoskeleton, but are covered by a delicate chitinous cuticle (Wigglesworth, 1933a).

It is now possible to answer the questions posed in the introduction. Firstly, the anal papillae are not constant during an instar but grow steadily. The 'intra-instar' growth-rate is less than that of the thorax breadth, but this is adjusted at each ecdysis by a sudden 'inter-instar' increase in size, so that the over-all growth coefficient is slightly greater than that for the thorax breadth (1.02855 c.f. 1.0), although this difference is not mathematically significant.

Secondly, none of the six other measurements has an intra-instar growth-rate similar to that of the anal papillae, and therefore from this data it is not possible to arrive at an anal papilla index independent of growth. Marshall (1938) expresses the size of the anal papillae as a fraction of the saddle. The present author was not aware of this anal papilla/saddle index when he carried out his work, and consequently no measurements were made of the saddle. From the intra-instar growth coefficient of the anal papillae it seems unlikely that the saddle will have a similar growth coefficient, so that the anal papilla/saddle index is probably only an approximate correction for the growth of the anal papillae.

It is interesting to discover which of the two modes of growth, intra- or inter-instar, brings about the change of anal papilla length in solutions of low and high salinities.

VI. THE GROWTH OF THE DORSAL ANAL PAPILLAE IN VARIOUS DILUTIONS OF SEA-WATER

In order to minimize any genetic effect upon the length of the anal papillae, the eggs from several females were mixed and then divided into eight batches which were

reared under similar conditions other than the salinity of the water. Eight different salinities were chosen, ranging from distilled water (distilled in a copper still) to a solution of sea-water having a chloride content equivalent to a 1.1 per cent. solution of sodium chloride. In the solutions equivalent to 1.0 per cent. and 1.1 per cent. NaCl there was little difference in the anal papillae lengths, and as there was a larger mortality, owing to the high salinity, these results were amalgamated for the purposes of analysis. Apart from the fact that only fourth stage larvae were measured, the conditions remained the same as described in section III.

The results obtained are tabulated in Table X and are illustrated in fig. 15. In order to compare the lengths of the anal papillae of larvae reared in the different dilutions of sea-water the length of anal papillae, when the thorax breadth is $1,000\mu$, will be taken as a standard. In distilled water the papillae are very long (313μ), but in tap-water (= 0.006 per cent. NaCl) they are very much smaller (210μ). In sea-water equivalent to 0.1 per cent. NaCl the anal papillae are 190μ and they do not change as the salinity increases to 0.75 per cent. NaCl, but in 1.0 per cent. and 1.1 per cent. the papillae are distinctly shorter (152μ).

TABLE X
Growth of the dorsal anal papillae of *An. sergenti* in various dilutions of sea-water

Class no.	Percentage salinity						
	D.W.	T.W.	0.1%	0.25%	0.5%	0.75%	1.0 and 1.1%
i	a b c	17 673 172 (168)	18 683 148 (149)	31 685 146 (147)	12 698 146 (145)	No larvae	a 20 b 758 c 131 (131)
ii	a b c	24 772 177 (182)	35 775 162 (161)	53 770 157 (158)	21 772 156 (155)	17 771 161 (161)	
iii	a b c	24 863 294 (298)	45 853 191 (192)	35 864 173 (173)	52 859 169 (169)	31 857 164 (167)	25 849 173 (171)
iv	a b c	8 933 312 (306)	16 940 202 (203)	25 942 184 (183)	53 946 179 (179)	24 950 179 (180)	23 949 180 (183)
v	a b c	5 1,041 316 (318)	a 6 b 1,052 c 219 (216)	a 7 b 1,055 c 195 (196)	20 1,034 195 (188)	12 1,033 195 (191)	a 10 b 1,030 c 195 (193)
vi	a b c	No larvae		6 1,150 196 (201)	10 1,136 203 (204)		a 6 b 1,102 c 161 (160)
Mean percentage difference		1.0	1.5	0.5	1.3	1.1	1.0

D.W. = Distilled water. T.W. = Tap-water (salinity 0.006 per cent. NaCl).

a = Number of larvae in a class. b = Mean thorax breadth in μ . c = Mean length of the dorsal anal papillae in μ .

(123) = The calculated length of the dorsal anal papillae obtained by substituting the values of the thorax breadth (b) into expressions 19-25 given in the text. Where only one or two representatives of a class were measured they have been attached to the neighbouring class.

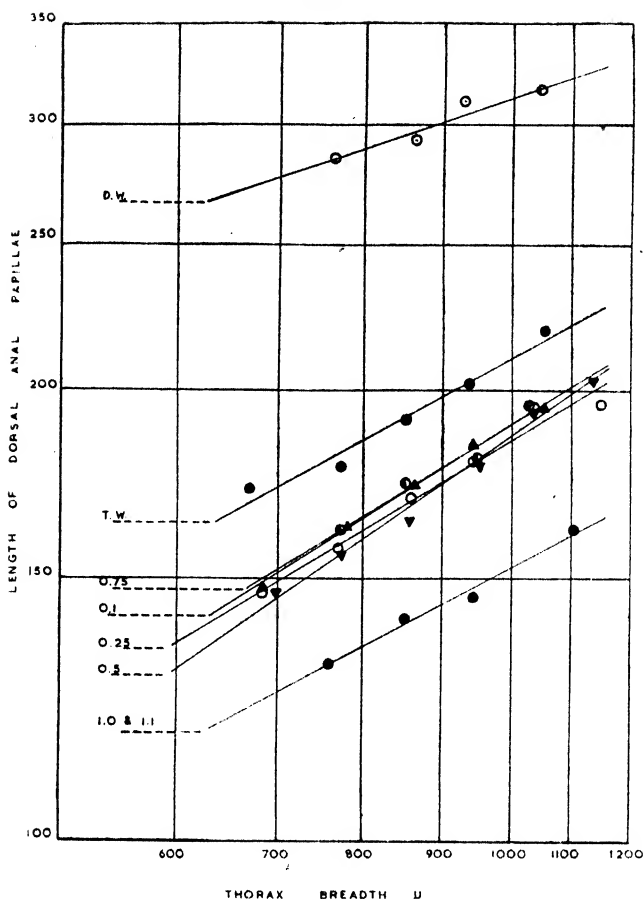


FIG. 15. The growth in length of the dorsal anal papillae of *An. sergenti* fourth stage larvae reared in diluted sea-water equivalent to various concentrations of NaCl solution. Open circles represent distilled water and 0.25 per cent. NaCl; closed circles represent tap-water and 1.0 and 1.1 per cent. NaCl; normal triangles represent 0.1 per cent. NaCl; inverted triangles represent 0.5 per cent. NaCl; half-closed circles represent 0.75 per cent. NaCl. Data from Table X. Logarithmic co-ordinates.

The intra-instar growth of the anal papillae of fourth instar larvae in the various salinities can be represented by the following expressions :

Distilled water	$\log y = 0.34699 \log x + \log 28.523,$	19
Tap-water	$\log y = 0.55604 \log x + \log 4.5053,$	20
0.1 per cent. NaCl	$\log y = 0.64138 \log x + \log 2.2599,$	21
0.25 per cent. NaCl	$\log y = 0.60459 \log x + \log 2.8361,$	22
0.5 per cent. NaCl	$\log y = 0.70162 \log x + \log 1.4634,$	23
0.75 per cent. NaCl	$\log y = 0.60899 \log x + \log 2.8173,$	24
1.0 and 1.1 per cent. NaCl	$\log y = 0.53862 \log x + \log 3.6008,$	25

and the intra-instar growth coefficients and their standard errors are :

Distilled water	0.34699 ± 0.08436
Tap-water	0.55604 ± 0.06376
0.1 per cent. NaCl	0.64138 ± 0.02799
0.25 per cent. NaCl	0.60459 ± 0.05187
0.5 per cent. NaCl	0.70162 ± 0.03395
0.75 per cent. NaCl	0.60899 ± 0.07786
1.0 and 1.1 per cent. NaCl	0.53826 ± 0.03310

Mathematically, a few of the differences between these intra-instar growth-rates are significant: e.g., between the growth-rates of the anal papillae in distilled water (D.W.) and in 0.5 per cent. NaCl, $P < 0.01$; between D.W. and 0.25 per cent. NaCl, $P = 0.05$; between 0.5 per cent. NaCl and 1.0 and 1.1 per cent. NaCl, $P = 0.02-0.05$. However, few of the differences are statistically significant. For our present purpose it is sufficient to note that the intra-instar growth-rate bears no relation to the length of the anal papillae. Thus the lowest intra-instar growth-rate is found in distilled water, when the anal papillae are the longest, and the intra-instar growth-rates of the anal papillae in tap-water and in 1.0 and 1.1 per cent. NaCl are almost the same, yet there is a very marked difference in their lengths. If the intra-instar growth-rate of the fourth stage is representative of those of the earlier stages, it follows that the length of the anal papillae is determined by the inter-instar growth at ecdysis.

The nature of the change which takes place in the anal papillae of larvae reared in waters of different salinities is not fully understood. The growth partition coefficient implies that there is a constant ratio of increase between the various parts of the body, that is, when a certain amount of raw material (product of digestion) is available for increase of cell size (Abercrombie (1936) has shown that growth in insects is largely due to increase in cell size and not in cell number), then so much will be allotted to each part of the body. This increase in tissue size is usually reflected in an increase in linear measurements, and it is this which is often measured; yet the fundamental meaning of the growth partition coefficient is a constant ratio of increase in cellular material (i.e., weight), not in linear dimensions.

The anal papillae are thin-walled finger-shaped protrusions lined with a single layer of flattened cells surrounding a large space filled with haemolymph. This haemocoel forms by far the largest part of the papillae, and the actual amount of cellular material in them is very small. It is therefore quite possible that the changes in papillae length may be brought about without any alteration in the volume of cellular material, but by a decrease or increase in the haemocoel.

In support of this interpretation of the enlargement of the anal papillae it is a matter of observation that their cellular lining is very thin when the papillae are hypertrophied and that it is thickened when they are stunted; but critical weighings are needed to determine whether the changes in anal papillae length are due to changes in the amount of cellular material in the papillae, that is, to alteration in the growth partition coefficient, or whether the amount of cellular material remains constant and only its outward expression alters.

VII. DISCUSSION

Wigglesworth (1933b) has pointed out that the anal papillae do not function as respiratory organs but are concerned with the intake of water. This conception has been modified by Koch (1938), who found that the anal papillae are able to absorb chloride from dilute solutions—a view confirmed by Wigglesworth (1938a). Thus, whilst the enlargement of the anal papillae in distilled water is regarded as a functional hypertrophy connected with the uptake of chloride ions, the nature of the mechanism which regulates the length of the anal papillae is not known.

Jobling (1938) has suggested that the length of the anal papillae in *C. pipiens* is dependent upon the osmotic pressure of the solution in which the larvae are reared. In

support of this it was stated that the mean anal papillae length of larvae reared in 0.75 per cent. NaCl at a mean temperature of 15° C. measured 0.45 mm., compared with 0.37 mm. when the temperature averaged 22° C. This difference was attributed to the effect of temperature on the osmotic pressure of the solution, but this explanation seems unlikely to be true, as the osmotic pressure of a 0.75 per cent. NaCl solution at 22° C. is equivalent to that of a 0.77 per cent. NaCl solution at 15° C., and it is difficult to believe that such a small change in osmotic pressure could produce such a relatively large reduction in anal papilla length. Moreover, from fig. 5 (Jobling, 1938) it appears that the anal papillae of larvae reared in 1.0 per cent. NaCl have an average length of 0.35 mm., compared with 0.45 mm. when reared in 0.75 per cent. NaCl. Clearly, the factor bringing about this change in anal papilla length with increasing temperature is not the osmotic pressure of the external medium.

It is probable that the difference in anal papilla length is due to the direct effect of temperature on the size of the larvae, rather than to its effect upon the osmotic pressure of the medium. Martini (1924) has shown that mosquitoes reared at high temperatures develop more rapidly but are smaller than those reared at low temperatures, and in confirmation Shannon and Hadjinaloo (1941) have found that autumn and spring adult mosquitoes are larger than summer adults. If this applies to *C. pipiens*, then larvae reared at 15° C. will be larger and consequently have longer anal papillae than larvae reared at 22° C.

The osmotic pressure of the haemolymph of *C.p. autogenicus* and *Ae. aegypti* has been shown to be due to a chloride fraction and a non-chloride fraction—probably amino acids (Wigglesworth, 1938a). Whilst the total osmotic pressure remains unchanged, the chloride fraction is subject to wide variation. It is low (0.12–0.16 per cent. NaCl) when the larvae are reared in distilled water, but in tap-water and concentrations up to 0.75 per cent. NaCl the chloride level is constant around 0.3 per cent. NaCl, whilst in concentrations between 0.75 and 0.9 per cent. NaCl the regulation of the chloride fraction breaks down and both the chloride fraction and the total osmotic pressure of the haemolymph begin to increase.

In section VI (see fig. 15) it was shown that the following relationship existed between the salinity of the external medium and the length of the dorsal anal papillae. In distilled water the anal papillae were very long, though they were markedly shorter when the larvae were reared in tap-water. As the salinity of the external medium was increased from 0.1 to 0.75 per cent. NaCl the anal papillae remained unchanged, but in 1.0 and 1.1 per cent. NaCl they were reduced. Thus the length of the dorsal anal papillae of *An. sergenti* follows a similar but inverse pattern to that of the concentration of chloride in the haemolymph of *C.p. autogenicus* and *Ae. aegypti*. It is reasonable to compare these three species, since their larvae are inhabitants of fresh water. The comparison suggests that either the length of the anal papillae is regulated by the chloride content of the haemolymph, or, what is more likely, the same mechanism regulates both.

This view fails to explain observations made on *C.p. autogenicus* by Wigglesworth (1938a) and Jobling (1938). Wigglesworth, measuring six larvae in each salinity and using balanced salt solutions, found that the anal papillae decreased in size in concentrations of salts up to 0.34 per cent. NaCl, whilst Jobling, using solutions of NaCl, found that the anal papillae of *C.p. autogenicus* decreased gradually in length in salinities from 0.1 to 1.1 per cent. NaCl. Neither of these authors allowed for intra-instar growth,

although Wigglesworth noticed the large individual variations (30–50 per cent.). Unpublished observations made on *C. pipiens** in Egypt by the present author suggested that the anal papillae, when corrected for intra-instar growth, behaved in the same manner as those of *An. sergenti*. This relationship between the anal papillae lengths of *C.p. autogenicus* reared in different salinities needs to be reinvestigated.

The salinities of 52 breeding-places of *An. sergenti* in nature were measured, and 25 were found to be below 0.1 per cent. NaCl (22 below 0.02 per cent. NaCl)—that is, in the range of salinity where small changes produce large differences in the lengths of the anal papillae. Therefore, although under certain circumstances the anal papilla length of *An. sergenti* may prove to be a useful specific character, it is unlikely to be a critical one.

VIII. SUMMARY

1. This paper is concerned with the analysis of the growth of laboratory-reared larvae of *Anopheles sergenti*, as revealed by measurements made on a total of 500 larvae, of which 125 were in each of the four instars.

2. Attention is drawn to a method of calculating accurately the growth coefficient and its standard error.

3. The seven measurements made on each larva are length and breadth of head, thorax and second abdominal segment and the length of the dorsal anal papillae.

4. The head shows discontinuous growth, with a dramatic increase in size at each ecdysis. The mean head breadths appear to obey Dyar's law with a ratio of increase 1.524, but it is also demonstrated that the determination of the head size may equally be regarded as controlled by a growth partition coefficient. Moreover, it is suggested that Dyar's law is merely a special case of this more general conception. Rather surprisingly the over-all growth coefficient between the head breadth and the thorax breadth is 1.0.

5. The thorax, growing continuously throughout larval life, undergoes no sudden change at ecdysis, but its growth is heterogonic, since its length increases more rapidly than its breadth (1.09 compared with 1.00).

6. The second abdominal segment grows continuously throughout larval life, the growth-rates of its breadth and length being the same (1.03) and differing significantly from those of the thorax length and breadth.

7. The dorsal anal papillae show a mixed pattern of growth, since they not only grow steadily, though slowly, during an instar, but also have a sudden increase at ecdysis. The

* In Egypt both races of *C. pipiens* occur, and since they interbreed in nature it was not certain which race was used in these experiments. It was probably *C.p. autogenicus*, for the females were all collected from native houses and the characters of the larval siphon, as is shown in the following table, compared more favourably with those given by Jobling for *C.p. autogenicus* than for *C.p. pipiens*.

	No. of larvae	Siphon index			Siphon length, in μ		
		Max.	Min.	Mean	Max.	Min.	Mean
<i>C. p. pipiens</i> (Jobling, 1938)	415	6.4	4.5	5.3	1,850	1,184	1,574
<i>C. p. autogenicus</i> (Jobling, 1938)	362	4.8	3.5	4.2	1,554	1,110	1,329
<i>C. pipiens</i> ? from Egypt	116	5.4	4.2	4.8	1,521	1,187	1,337

intra-instar growth-rate is low (about 0.6) and variable from instar to instar, but the over-all growth-rate (1.03) is of the same order as that of the second abdominal segment. It is suggested that the dorsal anal papillae length at ecdysis is controlled by a growth partition coefficient.

8. From the intra-instar growth-rates of the dorsal anal papillae length of fourth stage larvae reared in distilled water, tap-water (0.006 per cent. NaCl), 0.1 per cent., 0.25 per cent., 0.5 per cent., 0.75 per cent., 1.0 per cent. and 1.1 per cent. NaCl, it is shown that these bear no relation to the lengths of the anal papillae. Indeed, the intra-instar growth-rate is lowest when the anal papillae are longest.

9. It is suggested that the variation in the lengths of the anal papillae of larvae reared in different salinities is determined by the same mechanism as that which controls the chloride content of the haemolymph.

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A NOTE ON THE EFFECT OF HIGH TEMPERATURE ON THE PUPAL STAGE OF *GLOSSINA* IN RELATION TO THE TRANSMISSION-RATE OF TRYPANOSOMES

BY

T. A. M. NASH

(Entomologist, Sleeping Sickness Service, Nigerian Medical Department)

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In an interesting paper Burt (1946) has recently shown that the temperature to which pupae of *Glossina morsitans* are exposed may greatly influence the infectibility of the flies by *Trypanosoma rhodesiense*. He found that the infection-rate in flies which had emerged from pupae kept at approximately 86° F. (30° C.) was nearly three times greater than among flies from pupae kept under ordinary (cooler) laboratory conditions (Burt, 1946). In these the median temperature was always below 86° F., by values ranging from about 3.6° F. (2° C.) during the hottest months to over 14.4° F. (8° C.) during the coolest months (Burt, 1947).

Burt also found that the time taken for the completion of the trypanosome cycle in the fly was also significantly shorter in those from incubated pupae. Further, he found that transmission resulted with *all* the batches of flies from incubated pupae, whereas failure resulted with many of the batches of flies from pupae kept at cooler laboratory temperatures.

On page 26 of his earlier paper Burt inquires: 'What are the microclimates to which tsetse pupae are exposed in the field during the year? Are the pupae subjected to considerably higher temperatures during the hot dry season . . . ?' As I had collected data on both these subjects, I wrote to Dr. Burt, who has since asked me to write a note on the subject.

My work on the pupal microclimates was undertaken at Gadau, Nigeria, which lies near the hot, dry, northern limit of the range of *G. submorsitans*, the West African race of *G. morsitans*. I doubt whether, anywhere in Africa, the temperature of the pupal environment is likely to be greater than at Gadau, where, even in the centre of a forest island, shade temperatures of 106° F. (41.1° C.) were recorded at four feet above the ground surface.

My data on the temperature of the pupal environment refer to maximum and minimum temperatures at 1½ in. depth, read from thermometers buried in the breeding-grounds beside copper-gauze chambers which contained pupae (Nash, 1942). The mean monthly temperatures given below are the average of the mean monthly maximum and minimum readings.

Pupae were found in the dry season *in large numbers* in soils of which the mean monthly temperatures ranged from 69°–82° F. (20.6°–27.8° C.). Very few pupae, if any, were found in breeding-grounds when the mean temperature was over 82° F. The highest mean monthly temperature recorded was 84.5° F. (29.2° C.), with an absolute maximum of 96.8° F., but by that time the breeding-ground had been vacated—only two pupae were found, one of which was dead.

Since Burt was incubating pupae at 86° F. (30° C.), it would seem probable that he was employing a temperature which would never occur in the pupal environment. Nevertheless, his work is of great value, because it suggests the possibility that the higher the pupal temperature the greater is the transmissibility-rate amongst the emergent flies. The highest dry-season soil temperatures occur in Nigeria at the end of the late dry season when the first tornadoes occur. Judging from the soil maxima, the mean temperature must be much higher than at any time in the rains. (See Nash, 1939, for wet-season data.)

It may be argued that a depth of 1½ in. was excessive, although at Gadau this was a common depth for pupae. In order to find out whether the difference between soil temperature at ½ in. and at 1½ in. is great, I made some recordings last dry season in a breeding-ground of *G. palpalis* near Kaduna, which is near the northern limit for this species (admittedly Kaduna is considerably cooler than Gadau). At the ½ in. depth the mean temperature throughout the hot season of 1947 ranged from 73° to 79° F. (22·8°–26·1° C.), and at 1½ in. depth from 72·5° to 76·5° F. (22·5°–24·7° C.); hence the greatest difference between the two depths was only 2·5° F., or 1·4° C.

I suggest that it is of considerable importance for Burt's work to be repeated, comparing the transmissibility-rate amongst *G. morsitans* bred from pupae incubated at 68° F. (20° C.) with that for flies bred from pupae incubated at 82° F. (28° C.). We know that this is a temperature range which occurs in the field in *G. submorsitans* breeding-grounds, and, as my previous work suggested that *G. submorsitans* and *G. morsitans* were physiologically very similar, the results would probably hold good for the species as a whole (Nash, 1937). Buxton and Lewis (1934) concluded from their laboratory experiments at Gadau that 75° F. (24° C.) is the optimum constant temperature for the pupae of *G. submorsitans*; it should be noted that this falls exactly mid-way in the range quoted by me.

Burt also deals with the literature on the subject of high temperature in association with increased transmission, and notes that Vanderplank found that the transmissibility of *T. congolense* was higher in both *G. morsitans* and *G. swynnertoni* when flies from incubated pupae were used. Burt quotes the work of other authors, which suggests that subjecting the adult fly to high temperature may increase the infection-rate, especially at the time of the infecting feed.

The work quoted above supports my tentative conclusions that within the savannah zone of West Africa the bulk of the sleeping sickness, which is carried by *G. palpalis* and *G. tachinoides*, is transmitted in the late dry season. This may not be the case in the relatively small areas of country, transitional from forest to savannah, where, in the rains, tsetse tend to enter the villages; but I believe that it is true of the vast areas in which the streams slowly dry up and the tsetse concentrate by the permanent pools at the village water-holes.

• I have been working for the last three years near the northern limit of *G. palpalis*, and have been studying seasonal movement and longevity by means of marked flies. The results demonstrate very clearly that, with rising evaporation and temperature and the drying up of the stream, the tsetse population moves up to the vicinity of some permanent pools; in the hottest month, at the very end of the dry season, concentration is complete, and miles of stream are evacuated. The surviving flies do not wander from these pools but remain beside them for some months, and do not recolonize the rest of the stream until the rains are well advanced. Very frequently villages are sited by such permanent

pools, thus giving rise to the closest of personal man-fly contacts, in which a few flies are immobilized by climate and perforce feed repeatedly on the villagers when they come for water, washing, etc.

My results also show that—certainly in normal and mild years—longevity in the hot weather is adequate to permit of the trypanosomes reaching the salivary glands, as well as permitting a long span of subsequent life in which the disease can be transmitted. The females live considerably longer than the males and must therefore be considered to be the more dangerous sex. Only in exceptional dry seasons, when the desiccating effect of the Harmattan wind is prolonged and temperature is high, does the evidence suggest that longevity may be inadequate to permit of transmission. When I have completed four or five years of observation, I hope to be able to publish figures on the seasonal changes in the longevity of *G. palpalis*.

Unfortunately it is very difficult to get confirmatory evidence from sleeping sickness dispensaries as to infection being of a seasonal nature. In the mild form of the disease, which is so common in Nigeria, the onset of serious symptoms may be greatly delayed. Again, when a man is busy farming during the rains, he may delay going to a dispensary until he is too ill to work, whereas in the slack dry season he may report soon after the first symptoms have appeared.

Should it be possible to prove my contention that the bulk of sleeping sickness is carried at the end of the dry season, it might prove to be a discovery of considerable economic importance. At present the lengths of the clearings of riverine vegetation on either side of village water-holes and fords are designed to prevent tsetse from reaching such contact-points in *both* dry and wet seasons. Since tsetse cross clearings much more readily in the rains, and will cross greater distances at that season, the length of clearings has to be increased so as to reduce wet-season man-fly contact. But if such contact is of no importance, because at that season of travel the tsetse at the ends of the clearings are composed of ever-changing individuals, and because low temperature leads to reduced infectibility and transmissibility, then it would be possible to reduce the lengths of clearings so that they sufficed to give protection in the late dry season only.

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THE PATHOLOGY OF AN EXPERIMENTAL AMOEBIIC INFECTION IN THE RAT

BY

G. T. STEWART*

(*From the Liverpool School of Tropical Medicine*)

AND

W. R. JONES

(*From Imperial Chemical Industries Limited, Biological Laboratories,
Hexagon House, Manchester*)

(*Received for publication October 30th, 1947*)

The pathological changes wrought by advanced amoebiasis have often been described, but the earlier changes, and more especially the factors influencing them, are less familiar. Our ideas regarding the early stages of the disease are derived from theoretical considerations and from experimental infections, together with a few instances in which early lesions have been detected at autopsy in man. In particular, the factors governing the peculiar clinical features of amoebiasis—the variations in host-susceptibility and the tendency to relapse—are still mysterious.

Experimental infections have been successfully induced in a variety of animals: in dogs (Löscher, 1875), cats (Kartulis, 1891), kittens (Kruse and Pasquale, 1894), rats (Lynch, 1915) and monkeys (Dobell, 1931); and the classical experiments of Walker and Sellards (1913) showed that man was also susceptible to experimental infection. Baetjer and Sellards (1914) and Chatton (1917) claimed to have succeeded in infecting guinea-pigs, but most workers have found this animal and the rabbit refractory to the infection.

In the induction of these infections, various methods have been employed, each with a measure of success. The natural method of feeding cysts has given variable results in the hands of most workers, and greater success has attended the rectal inoculation of faeces containing trophozoites of *Entamoeba histolytica*, or the intracaecal inoculation of a culture (Meleney and Frye, 1936).

The experimental infection varies in different animals in two respects: firstly, in the proportion of animals capable of being infected—a variation influenced not only by biological factors but also to a considerable extent by the technique employed; secondly, in the course of the resulting disease. Thus in kittens, which are highly susceptible, the infection spreads acutely and is usually fatal. In dogs, the infection may follow a chronic course with the passage of cysts in the faeces, very much as in the human disease (Faust, 1932). A similar chronic disease has also been described in the monkey, but confusion has been caused by natural infections with *E. histolytica*, which are common in this animal (Dobell, 1931; Craig, 1934).

* Working at the direction of the Colonial Medical Research Committee.

Our own observations suggest that in the artificially infected young rat the course of the disease is intermediate; the onset is acute, but the disease is not usually fatal and various degrees of spontaneous remission commonly occur. In a few instances chronic lesions are established and occasionally cysts may be found in the faeces after 14–28 days.

In the past, *E. histolytica* infections in rats have been studied by several workers, with somewhat conflicting results. Natural infections were noted by Lynch (1915) and Atchley (1936) in wild rats but not in laboratory rats. Lynch (1915), Kessel (1923), Chiang (1925), Tsuchiya (1939) and Böe (1939) succeeded in infecting laboratory rats but failed to prove that tissue invasion had occurred, though Lynch and Kessel found ulceration in a few animals.

The present studies were based upon an experimental technique, previously described by one of us (Jones, 1946), in which invasion of the caecal wall by *E. histolytica* could clearly be demonstrated in histological sections. In a proportion of animals this tissue invasion was sufficiently massive to produce gross ulceration visible to the naked eye. Infection was secured in 67–90 per cent. of animals and there was no evidence that even a fraction of this percentage coincided with a natural infection. Not infrequently we noted the presence of one or more species of *Entamoeba*, which may possibly be enzootic in some varieties of rats (Chiang, 1925), but there was no evidence that these organisms invaded the tissues. A characteristic species of *Trichomonas* was regularly present and was occasionally associated with mild superficial inflammation and frothy caecal contents.

With this technique, the rats can be infected in numbers sufficient for statistical analysis of some of the factors governing the induction and course of the experimental disease. There is reason to believe (see below) that the factors investigated have some bearing on the problems of amoebiasis in man.

Design of the Experiments

The experimental methods described below were designed to study the following aspects of the pathogenesis of amoebiasis:

1. The relationship between infectivity and virulence of *E. histolytica*.
2. The histology of the development, course and healing of the lesions.
3. The influence of indigenous and added bacteria.
4. Chemotherapeutic protection.

METHODS

1. *Cultures of E. histolytica*

The amoebae were obtained from cysts in human faeces; the cysts were concentrated in sugar solutions by the method of Yorke and Adams (1926) and allowed to excyst in a liquid medium of the following composition (Jones, 1946):

Sterile horse serum	0.5 c.cm.
1 per cent. Marmite solution (aqueous)	1.0 c.cm.
Phosphate-buffered saline (pH 7.2)	8.5 c.cm.
Rice starch	30 mgm.

Stock cultures showing vigorous growth were transferred to 150 c.cm. of the same medium in conical flasks, and the inoculum was prepared from this after incubation at 37° C. for two days.

2. *Amoebic Inoculum*

The amoebae were concentrated by centrifuging the deposit from several flasks and mixing the centrifuged deposit with an equal volume of 10 per cent. gastric mucin. The inoculum used in most of the experiments was 0.2 c.cm. of this mucinous suspension, containing up to 300,000 trophozoites. This inoculum always included one or more strains of bacteria present in the original amoebic culture, since, as has long been known, growth of *E. histolytica* depends in some way upon the presence of bacteria (Chinn *et al.*, 1942). To investigate the influence of other bacteria on the amoebic infection, broth cultures of various bacteria were added to the suspension of amoebae; the inoculum was adjusted so that the control and test groups received like numbers of *E. histolytica* and the same volume of inoculum intracaecally.

3. *Operative Technique*

Four-weeks-old rats evenly matched in weight (20–35 gm.) were arranged in control and test groups. Under anaesthesia (ether and trilene) the caecum was mobilized through a small laparotomy wound. The inoculum, kept at 37° C., was injected intracaecally and the abdomen closed. 'Blank' operations showed that the operative technique was attended by a negligible mortality (less than 1 per cent.) and that it did not affect the animal adversely.

4. *Technique Used in the Study of Added Bacteria*

Broth cultures of bacteria, isolated from human faeces, were added to the amoebic inoculum or fed orally to the rats before or after the establishment of the amoebic infection. Heavy doses, ranging from 10 to 1,000 million bacteria, were administered, representing 0.1 c.cm. of intracaecal and 1 c.cm. of oral inoculum. As controls, various groups of animals received like numbers of living or killed bacteria, with or without the amoebic inoculum. *In vitro* tests were also conducted by adding bacteria to growing cultures or suspensions of amoebae.

The intestinal flora of control and infected rats were studied by plating caecal contents taken *post mortem*. As a routine, the following media were employed: horse-blood, McConkey, desoxycholate-citrate, bismuth sulphite agar and blood agar containing 0.1 per cent. sodium azide; anaerobic cultures were not used.

5. *Chemotherapy*

In certain experiments, rats were dosed with phthalyl sulphathiazole (500 mgm./kgm. orally) or with penicillin (500 units subcutaneously). In each case four doses were given in the 48–54 hours before or after the operation. These concentrations have no effect upon *E. histolytica in vitro*, though it is likely that they exert a considerable effect upon the coliform bacterial flora (Stewart, 1947b).

6. *Assessment of Results*

After periods ranging from one to 14 days, the animals were killed by chloroform and examined. The caecum was removed, cleared of faeces and inspected. Smears and sections were made for microscopic examination. Where bacteriological examinations were being conducted, post-mortems were performed under aseptic conditions.

Graphs showing the relationship between the infectivity and the virulence of *E. histolytica* in groups of experimentally infected rats.
Each point represents a group of 8-20 rats.

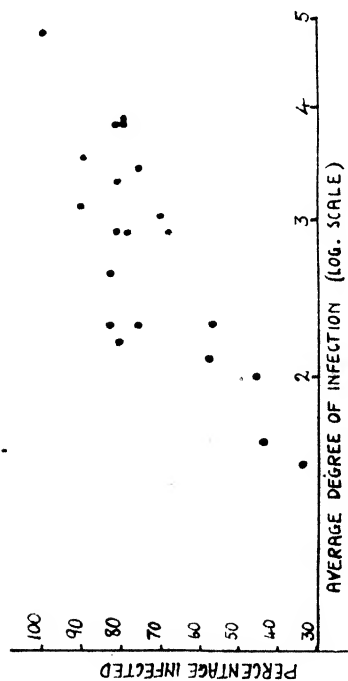


FIG. 1. *E. histolytica* strain W.
Correlation coefficient = 0.8082 (21 pairs). $P = 0.001$.

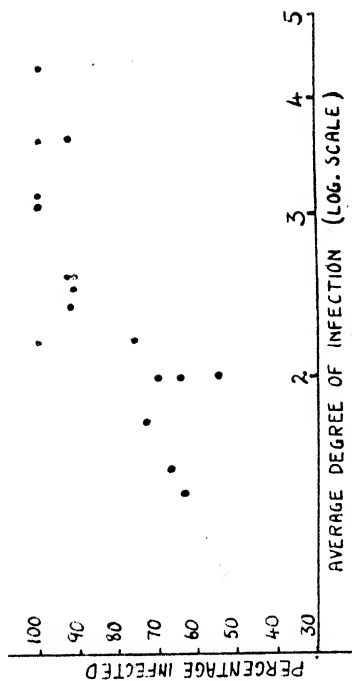


FIG. 2. *E. histolytica* strain P.1. Original experiments.
Correlation coefficient = 0.7731 (17 pairs). $P = 0.001$.

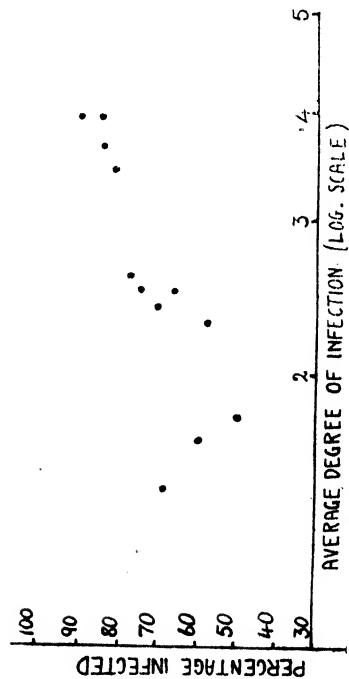


FIG. 3. *E. histolytica* strain P.1. After 6 months.
Correlation coefficient = 0.8784 (12 pairs). $P = 0.001$.

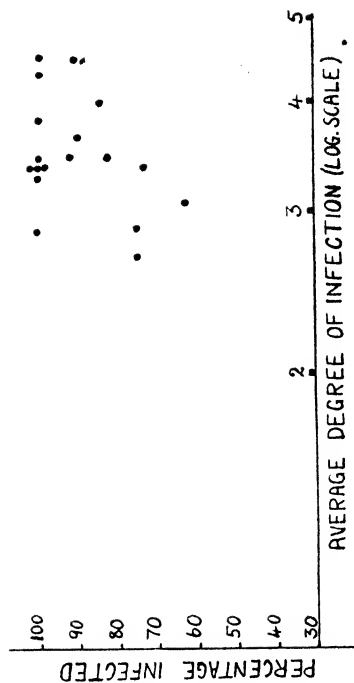


FIG. 4. *E. histolytica* strain M.1.
Correlation coefficient = 0.2726 (18 pairs). $P > 0.1$.

Two quantitative criteria of infection were employed :

(a) The average degree of infection (A.D.I.) of each group was calculated as the arithmetic mean of the symbols (shown below) for each animal in the group.

Heavy infection, ulceration ; numerous amoebae	...	5
Inflammation and mucus ; numerous amoebae	...	4
Inflammation ; many amoebae	...	3
Normal ; many amoebae	...	2
Normal ; few amoebae	...	1
Normal ; no amoebae	...	0

(b) The percentage of animals infected in each group was noted.

The following statistical formula was used to assess the significance of the differences in A.D.I. between two groups, x and y, containing numbers of rats m and n respectively.

$$z = \frac{x - y}{\sqrt{\frac{\sigma^2 x}{m} + \frac{\sigma^2 y}{n}}}$$

The standard deviations (σx and σy) were read from a curve plotted by one of us from a large number of groups, correlating the standard deviation of the variation of the degree of infection with the A.D.I. for each group (Jones, 1946).

RESULTS

Infectivity and Virulence of E. histolytica

The term 'infectivity' denotes the ability of the parasite to infect a susceptible host, and infectivity may be measured by the percentage of animals successfully infected in each group ; the virulence is indicated by the severity of the lesions produced and may be measured by the A.D.I. The relationship between the two is shown in figs. 1-4 for three strains of *E. histolytica*. When the percentage of animals infected in each group was graphed against the group A.D.I., two strains showed a linear relationship, suggesting that the infectivity of these strains was directly related to their virulence for the tissues of the host. The relationship was found to persist over many months of repeated sub-culture (fig. 3). A third strain (M.1) gave a high percentage of virulent infections with no linear relationship (fig. 4). The infectivity was to some extent independent of the number of amoebae in the inoculum (fig. 5). Passage experiments, conducted by infecting fresh groups of rats with trophozoites cultured from heavily infected rats, failed to endow the amoeba with increased infectivity. Since bacteria from the original lesions were necessarily transferred along with the amoeba, any alteration in virulence could not be assessed.

Tissue Invasion

The mucosa of the caecum showed evidence of invasion by *E. histolytica* within 24 hours after the intracaecal inoculation. This process began with minute erosions of the columnar epithelium in any part of the villus, a process which Craig (1927) and Westphal (1938) have attributed to cytolytic or proteolytic ferments secreted by the amoeba. Thereafter, two types of early lesions were observed :

(a) Invasion of the villi and crypts by amoebae, with some necrosis of the adjacent mucosa ; at a later stage, the mucosa at the edge of this nidus became heaped up and

formed a follicular ulcer, full of mucus and amoebae. Beyond the ulcer the mucosa remained healthy. Lesions of this type conformed to the picture of aseptic necrosis described by Westphal, and appeared to develop slowly (Plate I, photographs 1 and 2).

(b) A relatively wide-spread cellular infiltration by polymorphonuclear leucocytes of the tissue around the original breach in the mucosa. The bowel wall showed congestion and focal lymphoid hyperplasia. Amoebae were often scanty. Lesions of this type were especially prevalent when virulent bacteria were added to the inoculum, and probably represented a rapid invasion of tissue originally opened by the amoeba (Plate I, photograph 3).

Between these two types of lesions various intermediate changes were observed, and the subsequent changes showed features of both (Plate II, photograph 4). Mucus

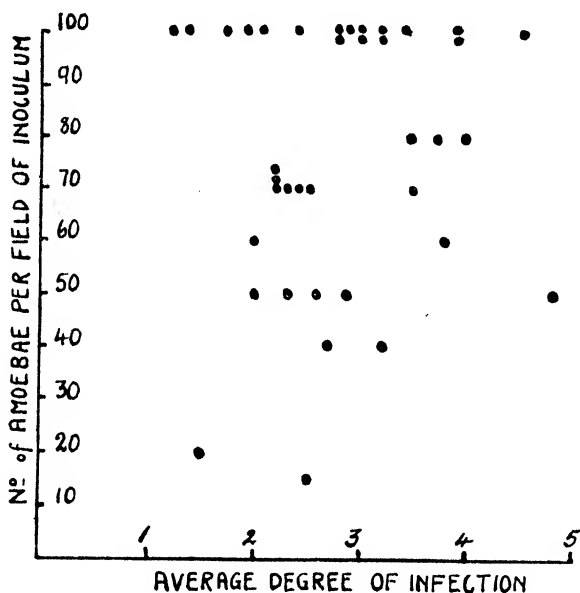
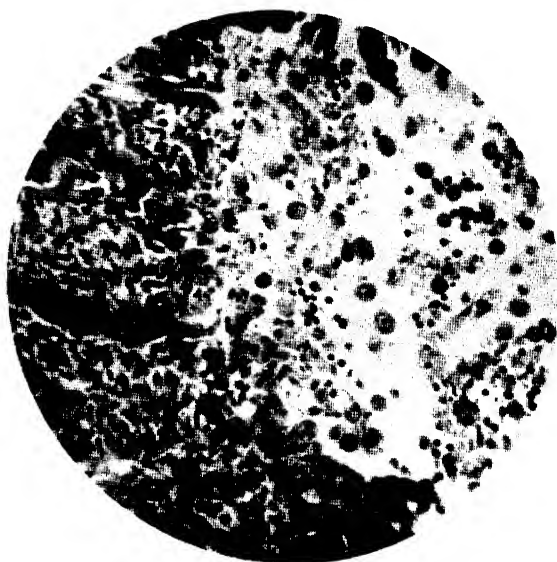


FIG. 5. Degrees of infection produced in the rat by different-sized inocula of *E. histolytica*.

secretion occurred at the site of the lesion, spreading outwards. The surrounding crypts were often filled with mucus containing numerous amoebae. In some animals the infection remained superficial, but in others the tips of the villi were destroyed and amoebae gained entrance to the submucosa, where they could be seen in tissue spaces and, very occasionally, in small blood-vessels. Extension of the invasive process led to wider undermining and desquamation of the mucosa, accompanied by interstitial oedema of all the layers of the bowel wall and by round-cell infiltration. In the later stages (3-7 days) the bowel was grossly ulcerated and thickened, with a copious exudation of mucus into the lumen and local peritonitis which often caused adhesion to the adjacent ileum or to the parietal peritoneum (Plate II, photograph 5). Amoebae were found chiefly at the bases of the ulcers or in the loose exudate, sometimes forming a solid mass; isolated amoebae were seen in the submucosa and, rarely, outside the muscularis mucosae.



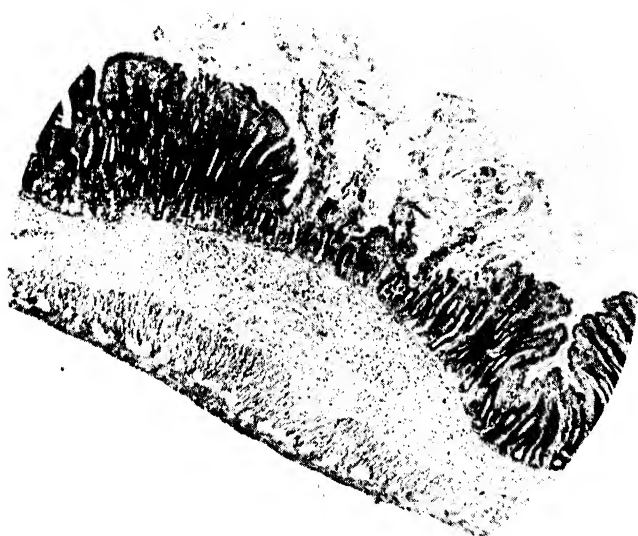
PHOTOGRAPH 1. Early destruction of the mucosa by *E. histolytica*. The lesion is localized and there is little or no inflammatory reaction. The adjacent mucosa is undamaged. ($\times 40$.)



PHOTOGRAPH 2. Same as photograph 1. ($\times 250$.)



PHOTOGRAPH 3. Secondary infection of an early amoebic lesion by *paracolon* bacteria. There is a wide-spread inflammatory reaction. ($\times 40$.)



PHOTOGRAPH 4. Superficial amoebic ulceration. The villi are half destroyed but the submucosa, muscular and peritoneal coats are undamaged. ($\times 40$.)



PHOTOGRAPH 5. Amoebic ulceration, with heavy bacterial infection. The mucosa is disrupted and there is a marked inflammatory reaction in the peritoneal coat. ($\times 40$.)

Healing followed the usual mechanism of repair after acute inflammation: the ulcer base became filled with a mound of cellular granulations, while epithelial regeneration began at the edges and covered the deficiency. There was comparatively little fibrosis or residual thickening. Healing could begin at any time after the initial amoebic invasion, but usually it seemed to assert itself after seven days. In some cases the healing process was retarded and the infection persisted for a few weeks; it is possible that this was partly attributable to a heavy secondary (bacterial) infection, since tissue invasion was often aggravated by the addition of virulent bacteria, as described below.

When tissue invasion occurred, the animals lost weight and occasionally death occurred after 3-4 days from severe amoebic infection. Most fatalities, however, occurred during the first 24 hours and were associated with a coliform bacteraemia. The operative technique probably permitted some leakage of the inoculum from the caecum, and in the first 24 hours coliform bacteria could often be cultured from the peritoneal cavity. During this period a transient bacteraemia was common, and a small proportion of the animals may have succumbed to it. When virulent bacteria were added to the inoculum, the death-rate increased and the appropriate organisms could be recovered from the heart-blood and spleen (see below).

No amoebae were observed in liver sections taken at various stages of the infection, but minor abnormalities were observed in the liver in a few animals; these changes consisted of congestion, periportal infiltration by round cells, and focal necrosis. Since similar histological appearances were obtained when bacteria were injected intraperitoneally, it is probable that the liver changes in the experimental infection in the rat were attributable to bacteraemia.

Evaluation of the Rôle Played by Bacteria

Cultures of *E. histolytica* trophozoites could not be grown free from bacteria, and for this reason the inoculum always contained one or more species of bacteria—usually *Bact. coli* and enterococci—derived from the host yielding the particular strain of amoeba. These, together with the bacteria already present in the rat's intestine, may well have played a rôle in every infection; in any event, they were inseparable from the experimental conditions.

Rats successfully infected in the usual way showed very few abnormalities in the bacterial flora of the caecum. The composition of this flora in the normal rat is shown in Table I, together with a summary of isolations made from infected rats. The only significant change was the predominance of non-lactose fermenters in a proportion (16 per cent.) of the infected animals. Organisms of this type were present in about 21 per cent. of normal rats. When such animals became infected with *E. histolytica*, it is possible that pathogenic non-lactose fermenters acted as potent secondary invaders in the amoebic lesions and thus became more prevalent in cultures made from the infected bowel.

Variable results were obtained when bacteria were added to the inoculum or administered by mouth to infected rats. Certain bacteria exhibited a qualitative effect, in that established amoebic lesions were rendered more severe. Sections in these cases showed severe ulceration and thickening of the bowel (see Plates I and II), with a considerable exudation of muco-pus. This effect did not raise the A.D.I. significantly, since the change affected only those animals in which amoebic infection was already established, and the A.D.I. afforded no measurement of the extent of ulceration in individual animals (Table II).

TABLE I
Composition of the intestinal bacterial flora in infected and uninfected rats

Description of group	No. in group	Intestinal flora					
		<i>Bact. coli</i>	<i>aerogenes</i>	Enterococci		Non-lactose fermenters†	
				Predominant	Present	Predominant	Present
Uninfected; caecum normal ...	42	41	23	16	26	—	9
Infected with <i>E. histolytica</i> ; A.D.I.* 3-5	37	32	11	21	16	6	8

$P < 0.02$

* A.D.I. = Average degree of infection, arrived at by taking the arithmetic mean of the following symbols for each animal in the group:

Heavy infection, ulceration; numerous amoebae ...	5
Inflammation and mucus; numerous amoebae ...	4
Inflammation; many amoebae ...	3
Normal; many amoebae ...	2
Normal; few amoebae ...	1
Normal; no amoebae ...	0

† P = The probability of a given result occurring by chance (Fisher and Yates, 1943).

‡ Non-lactose fermenters identified: saccharose-fermenting *paracolon* bacteria; *Proteus*; *Morgan*.

Aggravation of the lesions was most pronounced with *Bact. coli* and *paracolon* bacteria. Correlated with this, the identical strains of *paracolon* could be recovered from the caecum at autopsy of such animals. The *paracolon* strains used in these experiments belonged to group I (Sevitt, 1945). Although other *paracolon* strains were not uncommon in the intestinal flora of our rats, no group I strains were isolated except where a culture of these organisms had been administered to the rats. Hence it could reasonably be assumed that the strains used were recovered in accordance with Koch's postulates. With one strain an additional check was afforded by preparing an antiserum (rabbit). The organism under examination was agglutinated by this serum and absorbed the agglutinins for the type-organism. When *paracolon* bacteria were fed to uninfected rats, they remained present in the bowel for several days but caused no damage beyond slight hyperaemia.

TABLE II
The effect of bacteria in rats infected with *E. histolytica*

Control groups		Test groups				P
No. infected	A.D.I.	Organism	Method and time of dosing	No. infected	A.D.I.	
42/63	2.54	<i>paracolon</i> i	Intracaecal, 0 hours	36/58	2.12	0.29
47/69	2.55	"	Oral, 1-5 days	67/83	3.01	0.13
5/11	1.8	" ii	" "	5/8	2.8	—
		" iii	" "	8/11	2.1	—
14/23	2.56	<i>aerogenes</i>	Intracaecal, 0 hours	8/19	1.58	0.13
5/11	1.8	<i>Sh. flexneri</i>	Oral, 1-2 days	5/8	2.5	0.49
36/55	2.67	<i>Bact. coli</i>	" 1-5 "	45/60	3.17	0.21

Attempts at recovery were unsuccessful with *Shigella flexneri*, an organism which failed appreciably to aggravate the infection. Recovery of added strains of *Bact. coli* was not feasible, since strains with identical reactions were already present in the rat's intestine. One added strain, however, proved capable of identification, by the spontaneous acquisition of unusual haemolytic powers after its sojourn in the rat's tissues. *Bact. aerogenes* also was occasionally present in the intestinal flora of rats, but the strains used failed to gain further prevalence when introduced artificially.

When bacteria were injected intracaecally, there was usually an increase in the death-rate of the rats, depending upon the lethal virulence of the organisms used. This effect was diminished when the bacteria were fed orally at varying intervals after the amoebic inoculum had been injected intracaecally.

Passage experiments, conducted with a *paracolon* strain, lowered the killing dose but did not endow the organism with added virulence for the colon. Similarly, a strain recovered from heavily infected lesions did not show any increase in local virulence.

Chemotherapeutic Protection

In the present studies, chemotherapy of the bacterial element in the experimental infection was attempted by the use of penicillin and phthalyl sulphathiazole. In the concentration used, neither substance showed any action *in vitro* on various strains of *E. histolytica*; hence it is likely that any action *in vivo* would be attributable to the anti-bacterial properties of the two drugs.

TABLE III

The effect of penicillin and phthalyl sulphathiazole in the experimental infection in rats

Control groups		Treated groups			P
No. infected	A.D.I.	Treatment	No. infected	A.D.I.	
PROPHYLACTIC : -40, -24, -18, -1 HOURS BEFORE INOCULATION WITH <i>E. histolytica</i>					
11/17	2.6	Sulpha.	7/21	1.3	0.05
		Pen.	3/19	0.68	0.01
		Pen./sulpha.	2/17	0.4	0.01
15/20	3.0	Pen./sulpha.	7/17	1.7	0.06
THERAPEUTIC : 24, 30, 48, 54 HOURS AFTER INOCULATION WITH <i>E. histolytica</i>					
6/10	2.9	Sulpha.	7/9	3.1	—
		Pen.	2/9	0.9	0.03
		Pen./sulpha	1/11	0.5	0.01

Pen. = Penicillin, 500 units subcutaneously.

Sulpha. = Phthalyl sulphathiazole, 500 mgm./kgm. orally.

Prophylactic treatment with each substance separately during the 48 hours before the inoculation of *E. histolytica* caused a significant reduction in the A.D.I. (Table III). Penicillin showed greater activity than the sulphonamide, and possibly an additive effect was obtained when both drugs were administered (Stewart, 1947b). No definite change in the relative distribution of the organisms could be found in the intestinal flora of treated rats. It is possible, therefore, that a concentration of drug was established in the wall of the caecum sufficient in residue to combat the ingress of bacteria during the period of tissue invasion by the amoebae.

invade the tissues. Frye and Meleney (1933) showed that changes in the bacterial flora of cultures altered the infectivity of the amoeba, while Spector (1935) reported that streptococci and pneumococci aggravated the lesions. Deschiens (1938) demonstrated conclusively that certain bacteria (*S. typhi*, *S. paratyphi B* and *Bact. coli*) or their products increased both the infectivity and the pathogenicity of *E. histolytica*; the same author drew attention to the fact that an infective strain of amoebae always consisted of a complex of amoebae and bacteria, but he stated that neither his own experiments nor the literature had defined the rôle played by bacteria as being major or minor.

Our own studies suggest that the main factors concerned in the pathogenesis of the experimental infection in the young rat are the invasiveness of the amoeba and the virulence of the intestinal bacteria, especially *Bact. coli* and *paracolon*. The efficacy of penicillin and sulphonamide shows that bacteria play a major rôle at the onset and in the subsequent development of the infection. It is possible that variations in host-susceptibility and in the duration of the infection may be explained to some extent by differences in the bacterial flora. The studies shed no light, however, on the mechanism of remission and relapse, a feature of considerable importance in human amoebiasis.

SUMMARY

Under experimental conditions, young laboratory rats are susceptible to active infection of the caecum by human strains of *Entamoeba histolytica*.

The onset of this experimental infection is determined by the invasiveness of the particular strain of *E. histolytica*.

The subsequent course of the infection and the character of the lesions are governed largely by the activities of certain intestinal bacteria; the introduction of virulent strains of *Bact. coli* and *paracolon* aggravates the lesions.

The effect of bacteria becomes manifest as soon as *E. histolytica* establishes a breach in the mucosa. Given in prophylaxis, penicillin and sulphonamide inhibit the bacteria and thereby mitigate the infection; for the same reason, penicillin shows therapeutic activity.

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OBSERVATIONS ON THE USE OF CERCARIAL ANTIGEN IN THE DIAGNOSIS OF SCHISTOSOMIASIS

BY

D. M. BLAIR

AND

W. F. ROSS

(From the Schistosomiasis Research Laboratory, Salisbury, Southern Rhodesia)

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Cercarial antigens are now an accepted aid in the diagnosis of schistosomiasis. American workers, Oliver González and Pratt (1944) and Katzin and Most (1946), have described the method of preparation of an antigen prepared from *S. mansoni* cercariae and its use as a skin-test. The last-named authors have found the antigen to be of value not only in diagnosis, but also in the assessment of cure in treated cases. Alves and Blair (1946, 1947) have described their experience in the manufacture and use of a cercarial skin-test antigen in the diagnosis of schistosomiasis in Southern Rhodesia.

THE PREPARATION OF ANTIGEN

The American antigen is prepared from cercariae shed by snails infected with *S. mansoni*. Cercariae are stored as dried powder after having been spun down in a centrifuge. The liquid antigen is prepared from the dried powdered cercariae by a 1 : 5,000 dilution with 0.5 per cent. solution of carbol-saline. Southern Rhodesian antigen is prepared from mammalian cercariae (presumed to be those of *S. haematobium*) shed from naturally infected *Physopsis africana*. The cercariae are trapped on filter-paper, through which is poured the filtered pond-water in which the snails have been kept. A naked-eye assessment is made of the number of cercariae shed by one or two infected snails in each 3 in. by 1 in. tube, and in this way it is possible to arrive at a rough estimate of the number of cercariae—usually 12,000–15,000—that are trapped on each filter-paper. On filter-paper the antigen is easily stored. Liquid antigen is prepared by macerating impregnated filter-papers in 1 per cent. carbol-saline for 48 hours. The fluid is finally expressed and the antigen made ready for use by the addition of an equal quantity of normal saline. One per cent. carbol-saline for maceration is calculated at 1 c.cm. for 2,000 cercariae. Approximately 85 per cent. of the fluid is recovered. Therefore 10 c.cm. would be used to extract 20,000 cercariae, and 8.5 c.cm. would be recovered and diluted with normal saline to a total volume of 17 c.cm., and the final concentration of antigen would be the products of 1,000 cercariae per c.cm. As the average dose of antigen injected in each skin-test is about 0.03 c.cm., the antigenic stimulus is provided by the products of 33 cercariae.

Cawston (1947a, 1947b) has objected to the use of cercariae from 'wild' snails in the preparation of the Southern Rhodesian antigen on the grounds that the exact species of the cercariae being shed is not known. No claim is made that all the cercariae used in making the antigen are those of *S. haematobium*, although as far as possible only cercariae of mammalian type are used. It has never been claimed that the antigen is species-specific—in fact the American workers referred to above have used an *S. mansoni* cercarial antigen in the diagnosis of *S. japonica* infections. If cercariae of other species of *Schistosoma* are to be found in the Southern Rhodesian antigen, the practical results which have been achieved would indicate that genus or group specificity is all that is necessary. On the other hand, workers with little experience in the use of the antigen have stated that false positive reactions may be given by subjects harbouring other helminth parasites. In our experience a wide range of helminth infections, other than schistosomiasis, has been found in the examination of skin-test negative cases. This has also been borne out by Oliver González and Pratt (1944).

THE EVALUATION OF SKIN-TESTS

Katzin and Most (1946) have discussed their experience in the use of a cercarial antigen prepared by Oliver González and Pratt (1944) from *S. mansoni* cercariae. This was used in the diagnosis of *S. japonica* infections contracted by United States troops in the Philippine Islands. They recommend that the initial skin wheal produced should have a diameter of 4.0 mm., and they consider as a positive response a wheal of which the diameter has doubled in size—that is, to over 8 mm.

We have examined a series of cases with the cercarial antigen as a skin-test, and have measured the wheal diameter, latero-medially, at the time of injection and at five-minute intervals until 25 minutes after the time of injection. The group of positive reactors consists of one European, 17 Eur-African children and 21 African school-children. Their infections are 22 with *S. haematobium*, three with *S. mansoni*, six with double infections, and eight who are skin-test positive but in whom no eggs are being passed. The negative reactor group consists of two Europeans, one adult African and 33 African school-children.

From Table I a comparison can be made between skin-test wheal diameters (measured latero-medially) in groups of positive and negative reactors. There were 39 positive cases and 36 negative cases, and in each one a cercarial antigen wheal and a control wheal was produced. Measurements were made of the wheal diameters at the time of injection and at five-minute intervals thereafter up to 25 minutes. The smallest, the largest and the average diameter are recorded for each group and at each time interval. The relative increase in diameter of the antigen wheal is shown, as compared with its own control at each time interval.

It will be seen from the table that, in the actual measurements of the diameters, the antigen wheals in the positive reactor group increased in size compared with the control wheals up to a maximum size between 15 and 20 minutes from the initial injection. In the negative reactor group there is seen to be little alteration in size of the antigen or control wheals. It should be emphasized that the optimum time for measurement is at 15 minutes, as at 20 minutes, although the wheal may appear larger, the edge is not so easily defined and measurements consequently tend to be inaccurate. The relative increases in size show that, in the case of the positive group, at 15 minutes the wheal diameter has more than doubled, while in the negative group the wheal diameter remains

within 15 per cent. of the initial wheal. In the positive reactors there is a clear-cut increase in size over the original wheal diameter. We agree, therefore, with Katzin and Most (1946) that the wheal diameter should double in positive reactors—that is, provided that the initial injection causes a wheal of a diameter about 5 mm. It cannot be expected, for example, that an initial wheal of diameter 10 mm. should become over 20 mm. at 15 minutes.

TABLE I
Comparison of positive and negative skin-test wheal diameters

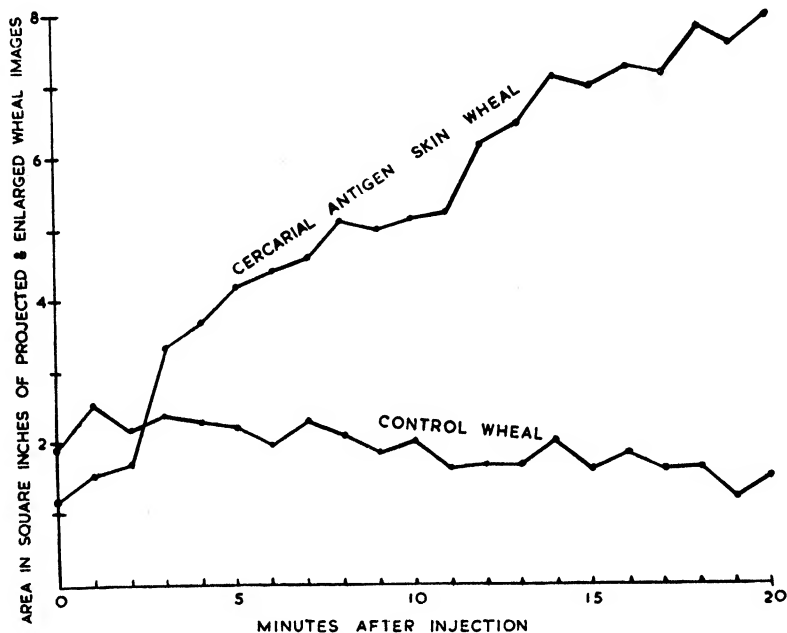
Time after injection, in minutes	Positive reactors (39 cases)				Negative reactors (36 cases)			
	Antigen wheal diameter, in mm.	Control wheal diameter, in mm.	$\frac{A}{C} \times 100$	Relative increase of antigen wheal	Antigen wheal diameter, in mm.	Control wheal diameter, in mm.	$\frac{A}{C} \times 100$	Relative increase of antigen wheal
0	3.00 4.77 7.00	3.50 4.85 8.50	98%	1	4.50 5.25 7.00	3.50 5.24 7.00	100%	1
5	3.50 7.70 12.00	3.00 4.78 8.50	161%	1.64	4.00 5.54 7.00	4.50 5.39 7.00	103%	1.03
10	6.50 10.05 21.00	2.50 4.62 8.50	217%	2.21	4.00 6.07 8.00	4.50 5.54 7.50	109%	1.09
15	8.50 10.67 21.00	2.50 4.78 7.50	223%	2.27	4.00 6.24 9.00	3.00 5.54 9.00	113%	1.13
20	8.50 11.56 20.50	2.50 4.44 6.50	260%	2.65	4.00 5.96 7.50	3.00 5.29 9.00	113%	1.13
25	7.50 10.17 20.00	2.50 4.19 6.50	243%	2.48	3.00 6.07 9.00	3.00 5.28 7.00	115%	1.15

In each column recording wheal-diameter measurements the minimum, average (in heavy type) and maximum diameters observed at each time interval are taken.

In our experience only a very small proportion of cases show a reaction which might be termed a doubtful positive. A doubtful positive reaction might be defined as one in which the wheal-diameter increase is between one and three-quarters and twice the original diameter.

Katzin and Most (1946) advocate the production of wheals 4 mm. in diameter, but we have found 5 mm. wheals easier to produce. A rough and ready measure of wheal diameter is to use the paper discs (confetti) produced by a paper punch, the diameter of these discs being approximately 6 mm. The wheal on injection should not be visible when covered by a disc, and at 15 minutes the positive wheal should be clearly seen in its whole circumference around the disc. It cannot be too strongly stressed that, if the original intradermal injection (which should be made with the proper equipment) is imperfect, another injection should be made on an adjacent site forthwith.

In one case a series of ciné photographs of the development of a cercarial antigen skin-test wheal and its control was made with the assistance of Mr. F. Goodliffe, Director of the Rhodesian Film Unit. Using a 35 mm. ciné camera, exposures were made of the development of the wheals at one-minute intervals. The film strip was then projected at a fixed enlargement on to squared paper, and the outline of each wheal was sketched. The area of each wheal picture outline, antigen and control, was calculated, and the result is shown in the accompanying fig. It can be seen from this that increase in the wheal size of the antigen begins early and is well advanced at five minutes. It must be pointed out that not all positive cases follow this pattern, and in some instances there may be relatively little increase in the size of the wheal until 10 minutes after the injection.



GRAPH showing the development of a cercarial antigen skin wheal and of a control wheal at one-minute intervals after injection.

THE STABILITY OF CERCARIAL ANTIGEN

Advantage has been taken of the presence of remaining stocks of several old batches of antigen to test their stability and keeping-powers. The various antigens were injected at one time into the arms of six skin-test positive cases, and the diameters of the wheals produced were measured at once and at 10 and 20 minutes after injection. The average wheal diameters produced by each antigen are shown in Table II. It will be seen that there is no significant difference in the reactions produced by the various antigens. It is interesting to note that the double-strength antigen (E in Table II) produces no greater wheal than the normal-strength antigens. It may be concluded that storage of antigen and its age after preparation in liquid form is of little practical importance. It should be remembered that antigen which has been stored in a refrigerator should be allowed to

warm to room-temperature before being injected, as there is a possibility of false positive reactions being stimulated in some individuals by the injection of a cold fluid.

TABLE II
Wheal comparisons with cercarial antigens

Antigen	Average wheal diameters, in mm. (6 cases)		
	On injection	At 10 minutes	At 20 minutes
A	5.4	11.5	12.9
B	5.2	9.6	10.3
C	5.3	9.6	10.9
D	5.6	11.1	12.5
E	6.0	11.3	12.6
F	4.9	4.75	4.2

A.—A miracidial antigen prepared in Salisbury in 1938 by Blackie and Alves. Eggs were obtained by scraping the mucosa of urinary bladders removed at post-mortem. The eggs were washed repeatedly and then crushed in a diluting fluid. After promising early results its use was abandoned because of the difficulties of preparation. A small supply in rubber-capped vials was left lying in a drawer until discovered in 1945. After that date they were stored in a refrigerator.

B.—Cercarial antigen made up in May, 1945, and stored in a refrigerator.

C.—Antigen from the same batch as B, but exposed for one year on a sunny window-sill in the laboratory.

D.—Cercarial antigen made up in July, 1947, and tested immediately.

E.—Antigen from the same batch as D, but double strength—that is, the final dilution with normal saline of the 1 per cent. carbol-saline extract was not made.

F.—Control fluid, 0.5 per cent. carbol-saline.

THE VALUE OF A CONTROL INJECTION

In the diagnosis of schistosomiasis in the individual patient, particularly in Europeans, it is wise to use a control. The interpretation of wheals in subjects who exhibit evidence of skin allergy is sometimes difficult, and the control injection may then be of assistance. No doubt there are a small number of people who are allergic to foreign proteins of diverse character, and whose skin, being unduly sensitive, may react positively to cercarial antigen or even to an injection prick without any intradermal injection.

In the African skin allergy is believed to be very rare, if it occurs at all, and in the testing of many hundreds of cases no assistance has been given by a control injection. It is considered that mass-diagnosis in Africans can be carried out with cercarial antigen alone, without a control injection. In dealing with large numbers of cases it is much easier to maintain a constant wheal size if only one syringe has to be handled.

SUMMARY

1. Recent work is described in the development of cercarial antigens for the diagnosis of schistosomiasis by a skin-test.

2. It is advocated that a positive skin-test be interpreted as one that doubles the diameter of the initial wheal in 15 minutes—that is, an initial wheal of 4–5 mm. should be over 8–10 mm. in 15 minutes.

3. Comparative tests of antigens prepared at various times show the keeping-properties to be good.

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LEISHMAN'S STAIN ADAPTED FOR USE WITH HISTOLOGICAL SECTIONS

BY

ROBERT H. BLACK*

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)

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The staining method here described was evolved in an attempt to discover an alternative method to that of Giemsa for the staining of tissue sections. The immediate problem was the staining of erythrocytic and exoerythrocytic forms of *Plasmodium gallinaceum*. A trial showed that Leishman's stain, used without preliminary treatment of the section, stained exoerythrocytic forms of *P. gallinaceum* well but erythrocytes poorly. Van Gieson was used as a preliminary stain and gave the effect desired. Picric acid was found to be the component of Van Gieson responsible for the improved results, while the fuchsin was shown to be deleterious. The mode of action of the picric acid was therefore studied.

The staining method was evolved for a specific purpose, and no extensive studies have been made of the results when it is used in the staining of various tissues and organs. However, sufficient tissues have been examined to give a general picture of its possible application.

METHOD

Thin paraffin sections, brought down to distilled water, are covered with a saturated aqueous solution of picric acid for 5–10 minutes. This results in a general light-yellow staining of the section. The picric acid is washed off with water and the section is covered with Leishman stain freshly diluted with twice its volume of distilled water. This is allowed to act for 25 minutes. The slide is then washed with running water for about 10–15 minutes. Excess water is removed and the section is dehydrated through the following series of xylol-acetone mixtures :

5 per cent. xylol, 95 per cent. acetone ;
30 per cent. xylol, 70 per cent. acetone ;
70 per cent. xylol, 30 per cent. acetone ;
Xylol.

The section is then mounted with a neutral mounting medium.

Van Gieson was used as the preliminary stain before its components were investigated separately. Two solutions were made up, one containing 1/20 per cent. acid fuchsin in water and the other being saturated aqueous picric acid. These solutions were used

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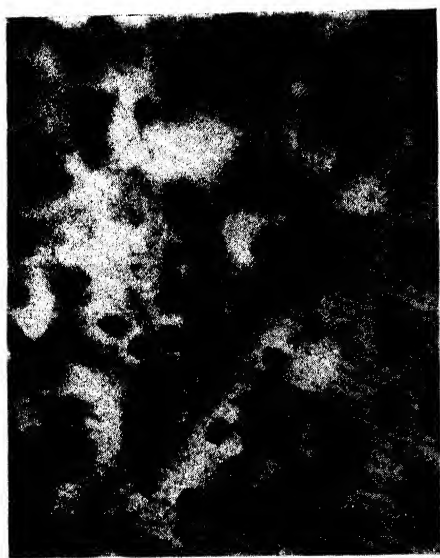


FIG. 1. Kidney, fowl, exoerythrocytic schizont of *P. gallinaceum*.



FIG. 2. Kidney, fowl, differential staining of collecting tubules.

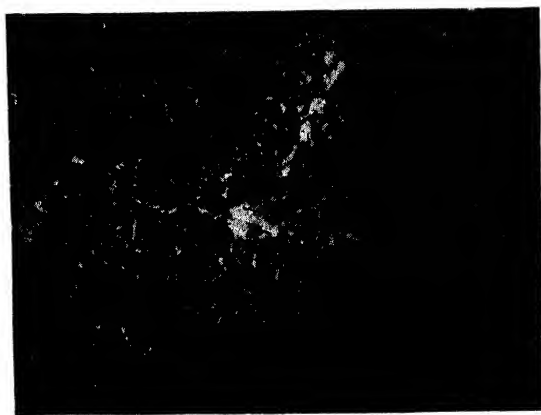


FIG. 3. Liver, monkey, central necrosis in infection with *P. knowlesi*.

separately as preliminary stains and were then followed by Leishman's stain. The results were compared with those obtained with Van Gieson as the preliminary stain. Fowl kidney was used as the test tissue. After the preliminary treatment with Van Gieson there was some faint staining of the mucous lining of the large collecting tubules; this effect was also seen with the fuchsin solution, but the picric acid stained the section uniformly light yellow. After staining with Leishman the sections preliminarily treated with Van Gieson and picric acid were stained satisfactorily, the cytoplasm of the erythrocytes being bright red. The picric acid gave a slightly crisper result. With fuchsin the final result was unsatisfactory—there was no differential tubular staining, the renal and blood-cells were of a blurred and indistinct purple colour, and, further, there was a fine deposit left on the section.

The mode of action of the picric acid was investigated. It was considered to act as a mordant and perhaps also by virtue of its acidity. The pH of the saturated aqueous picric acid used was 1.2. Deci-normal hydrochloric acid was substituted as the preliminary stain, and essentially similar results were obtained to those with picric acid. The effects of omitting the preliminary stain and of diluting the Leishman stain with water buffered to pH 6.0 were noted. The erythrocytes stained poorly and there was insufficient differentiation in the staining of the renal cells. The method was thus modified to use saturated aqueous picric acid as the preliminary stain, as the effect obtained with this acid appeared to be somewhat brighter than with hydrochloric acid.

RESULTS

The results obtained fall into three groups—the staining of normal tissues, the differential staining of some components of normal tissues, and the differential staining of parasites in tissue sections. The most striking results are obtained with cellular organs, such as the liver, kidney, intestine and spleen. In the liver the cells are stained brilliantly with an almost polychromatic effect. The blood-cells stain as with Leishman in a thin film, and malaria parasites in the red cells are easily recognized. Plate III, fig. 3, is a low-power view of liver showing central necrosis in *P. knowlesi* infection.

In the kidney of the fowl a differential staining effect is seen in the tubules: a magenta colour is seen on the internal surface in the collecting tubules (fig. 2), which is absent from the other tubules. In the fowl kidney this differentiation is also seen when Leishman's stain is used without preliminary acidification.

Exoerythrocytic forms of *P. gallinaceum* take on a much deeper stain than the rest of the sections (as seen in liver, spleen and kidney) and stand out readily, although there is no differential staining of the chromatin and cytoplasm of the parasites themselves. Fig. 1 shows an exoerythrocytic schizont of *P. gallinaceum* in kidney.

Erythrocytic malaria parasites, such as *falciparum* schizonts in sections of brain, are well stained by this method. If the section is not mounted in a neutral mounting medium the colour tends to fade from the section after some months.

COMMENT

The staining method evolved is not an improvement upon Giemsa stain at its best. It does, however, provide a method of staining tissues and some parasites in tissues, and is constant in its results. The preliminary treatment with picric acid appears to derive its effect from the acidity of the dye and probably also from a mordant effect.

A SIMPLE TECHNIQUE FOR THE MICROSCOPY OF LIVING TISSUES *IN SITU*, WITH SOME OBSERVATIONS ON THE SPLENIC CIRCULATION

BY

DAVID GALL

(From the Liverpool School of Tropical Medicine)

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HISTORICAL INTRODUCTION

The technique described in this paper is a modification of the transillumination technique of Knisely (1936*a*, 1937, 1938), and its historical background is virtually the history of the microscopy of living tissues.

The earliest work on the microscopy of living tissues, such as the observations of Malpighi (1686) on the circulation in the lungs and mesentery of frogs, and of Gruithuisen (1812) and Müller (1835) on the intact liver circulation, made use of strong reflected light directed on to the tissue. This method of illumination has been used in more recent times by Ghiron (1912) on the liver, by Krogh (1919) on various tissues, by Richards and Schmidt (1924) on the frog's liver, and by Olkon and Joannides (1930) on the pulmonary alveolar circulation of the dog, and indeed it is being developed along promising new lines at the present time (Barer, 1947).

Transmitted light from the substage of an ordinary microscope has also been long in use where the tissue to be examined could be suitably arranged on the stage—as in, for example, the work of Clark (1909 and subsequent papers) on the tails of amphibia, Zweifach (1934) on the capillaries of the frog, and McNee (1931) on the spleen. Modifications of this technique have mainly involved the method of preparation of the tissue concerned, as in the rabbit's ear chamber technique developed by Sandison (1924, 1928) and by Clark *et al.* (1930 and subsequent papers), and in the isolation of the kidney by Hill and McQueen (1921). Hall (1925), and MacGregor (1933) in a modification of Hall's method, dispensed with the microscope substage and studied the pulmonary alveolar circulation by focusing a filament source on the tissue by means of a flask condenser.

Meanwhile, the more mobile type of illumination given by internal reflection through a transparent rod was being developed. Basler (1917) quotes Kochs and Wolz (Fresenius, 1889) as being the first to use this method for microscopy, but Basler himself appears to have been the first to investigate the theory for microscopical purposes, and he designed an apparatus very similar in many ways to the one about to be described. He focused light from a filament source on to the end of a glass rod, suitably bent to reach the under-surface of the tissue and drawn out to a thin point. Light was internally reflected along the rod and directed upwards into the microscope by reflection from the bevelled under-surface of the tip. Loeffler and Nordmann (1925) used this apparatus for some extensive studies of the liver circulation, and, incidentally, they remark that they found reflected light as used by Gruithuisen, Müller and Ghiron unsatisfactory for solid tissues like the

liver. Florey and Carleton (1926) used a curved glass rod to direct light through the living cat's mesentery to study the capillaries while it was kept under physiological conditions in a warm bath. In 1935 Barta described an illuminator which consisted of a glass rod drawn out to a fine point, which was inserted deep into the tissue to be examined. With this he made observations on the living frog heart (Barta, 1935, 1936).

The use of fused quartz rods appears to have been introduced by Leiter (1925) and by Silverman (1925), but its first use for transillumination of living tissues appears to have been by Wearn *et al.* (1934), who directed light from an arc lamp into a narrow quartz cone and illuminated the lung *in situ* through the diaphragmatic pleura, viewing the circulation through the parietal pleura.

Only one record has been found of the use of plastic rods for transillumination—that of Williams (1944), who studied the thyroid isthmus in rats and mice by means of a Lucite rod inserted into the trachea.

It is to Knisely, however, that the major refinements and developments are due. His technique is described in his papers of 1936*a*, 1937 and 1938, and by its means he has made detailed studies of the splenic circulation (1936*b*, 1936*c*), the frog's liver (1939), and the circulation in malarious monkeys (Knisely, Stratman-Thomas and Eliot, 1941). Wakim (1942) and Wakim and Mann (1942*a*, 1942*b*) studied the liver circulation by the same method, and MacKenzie, Whipple and Wintersteiner (1940, 1941) have attempted to confirm Knisely's findings on the splenic circulation. Knisely's apparatus and some of the observations made with it will be described more fully below.

THE SCOPE OF THE TECHNIQUE

The present paper describes the evolution of a relatively simple technique, designed originally to contribute to the study of the circulation of the liver in malaria. Microscopy of the suitably illuminated living organ offered a direct approach to the problem. It had been amply shown that circulatory pattern, changes therein, and the site of such changes, could be studied visually at the time of their happening and under conditions approaching the physiological; but this approach has many natural limitations. Certain departures from the physiological seem to be inevitable: anaesthesia, exposure of the tissue, disturbance of pressure relations, and replacement of peritoneal or interstitial fluids by Tyrode's or Ringer's solution all seem difficult to avoid. Owing to the diffraction and absorption of light by the tissue, only the surface layers can be brought into focus, and only tissues less than a few millimetres thick can be adequately illuminated by transmitted light. This limits satisfactory observation to the smaller animals. Tissue-cells within their natural surroundings are not very refractile, and cellular detail is consequently difficult to make out. It takes some time to learn to recognize the architecture of a transilluminated tissue, and even after much practice interpretation of the phenomena seen remains partly subjective. These limitations and the attendant technical difficulties have been discussed by Knisely and by MacKenzie and his associates, and have been encountered again during the course of the present work; where necessary, they will be further discussed in their proper context.

In spite of such limitations the technique appears to be capable of wide application, and in the simple form here described has been used to study the spleen in some detail, as well as the liver. The work has now had to be brought temporarily to a close, and further modifications and simplifications are undoubtedly necessary.

DESCRIPTION OF THE APPARATUS

The apparatus is diagrammatically illustrated in the accompanying figure. The lamp consisted of a 6-volt 24-watt straight-coiled filament bulb, in a housing which incorporated a 50 mm. plano-convex condensing lens and a filter-carrier, the whole being mounted on an adjustable stand. By means of a home-made extension collar on the back of the lamp-housing the filament was brought to a focus about 15–20 cm. from the lamp. The lamp was set in such a way that this image lay in the plane of the thick end of the transilluminating rod. The filter-carrier held a circle of heat-absorbing glass (Chance Brothers, 'ON 19,' 3 mm. thick).

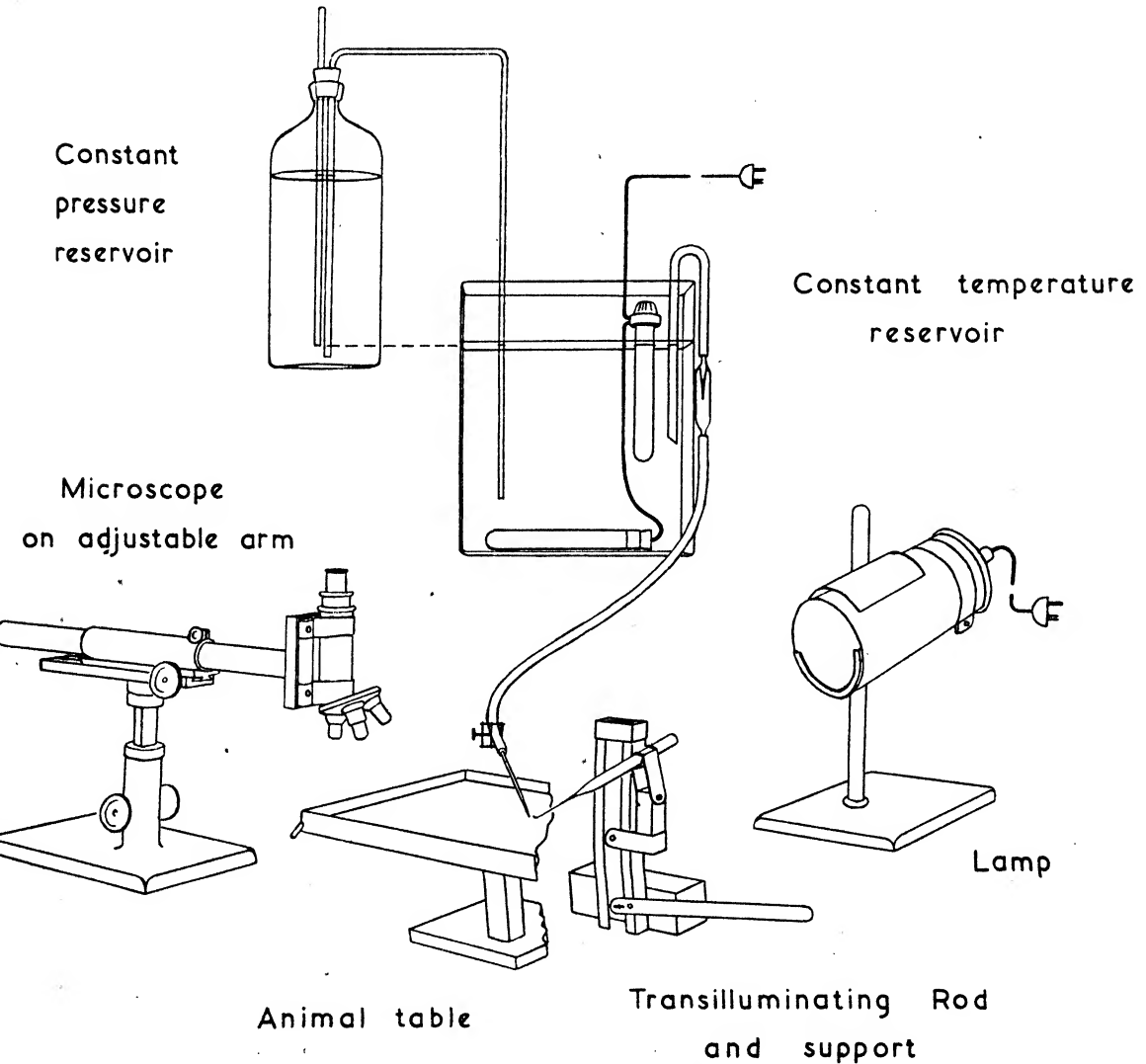


DIAGRAM showing the apparatus used.

The transilluminating rod was of ordinary laboratory-quality white glass, 6 mm. in diameter. Eight to 10 cm. from one end this rod was drawn out in the Bunsen flame into a thin portion of about the same length, the taper being made as gradual as possible. The end of this thin portion, which varied in diameter from about 0.5 to 2 mm., was then bent to a right angle, giving as wide a radius of curvature as was practicable. This radius of curvature was determined mainly by the amount of room available below the tissue to be illuminated. The unwanted glass beyond this bend was broken off. Facilities for optically grinding the ends of the rods were not easily available, although they would have been an advantage, but a clean break was found to give completely satisfactory results. Several of these rods were kept at hand, varying slightly in shape and size.

The rod was held in a simple home-made holder, which allowed easy movement about any axis and moderately fine adjustment vertically.

The animal table consisted of a waxed tin-plate tray with a drain from one corner, mounted on a pillar which allowed tilting in any direction. Space was left below for heating-lamps if required. The animal was supported clear of waste solutions on a suitable cork rest.

For the microscope there was fortunately available a Baker Universal stand, originally designed for use with a Greenough binocular microscope but quite simply adapted for use also with a Leitz monocular body. Both a binocular dissecting microscope and a monocular microscope were used, with a variety of objectives and eyepieces, the highest magnification being given by a Leitz 2 mm. water-immersion objective. The latter, in spite of slight loss of resolving power, was used over a cover-slip, as this made focusing easier by preventing the tissue from being drawn towards the objective by surface tension.

To keep the exposed and illuminated tissue at body temperature a flow of warm physiological solution (Tyrode was used, for abdominal organs) was allowed to bathe it. The apparatus for this consisted of a large reservoir bottle from which the contents were siphoned at constant pressure into a constant-temperature heating-bath. The constant-pressure head was maintained by stoppering the reservoir and allowing the replacing air to enter by a straight glass tube which opened near the bottom of the reservoir. Minor adjustments of the pressure head could be made by raising or lowering this air-inlet tube.

The constant-temperature bath was a flat glass tank supported about a foot above the level of the animal. A small coil heater lay on the bottom in relay with a thermostat. From this the solution was siphoned through rubber pressure tubing to a finely drawn-out delivery tube directed on to the tissue. The rate of flow was controlled by a screw clip. The temperature in the bath needed to be at a temperature rather higher than that of the animal, to allow for cooling of the solution in the delivery tube. To gauge the rate of flow a drip chamber was set in the delivery tube (close to the warm tank to reduce heat loss from the relatively thin-walled drip chamber).

SOME THEORETICAL POINTS CONCERNING THE APPARATUS

1. *Illumination*

The illumination given by this apparatus proved quite adequate for tissues up to about 2 mm. in thickness. The image of the bulb filament was about the same size as the end of the rod, and the light entered by a sufficiently narrow cone for most of the light to be internally reflected by the rod. As a source of light Knisely used projection-type

bulbs of 200–1,000 watts, which, though undoubtedly more efficient than the low-power bulbs used in the present apparatus, have disadvantages in that they require elaborate cooling and screening from the animal. Knisely used no condenser with these, and the rod, which was of quartz and some 30 cm. long, was brought as close to the filament as possible.

The use of glass in place of quartz appears to be justified. Although quartz has better transmitting powers and a slightly more suitable refractive index for internal reflection, the ease with which glass can be obtained and worked has advantages. The loss of light through a relatively short glass rod, and from imperfections within its substance, does not appear to affect its efficiency in practice.

A selection of rods is a necessity. Varying shapes and sizes help to overcome awkward access to the tissue, and—of even more importance—the area of the tips must be kept as small as the field of the objective (Barer, 1947). Light passing through the tissue is greatly scattered by refraction, reflection and diffraction, and by cutting down the area illuminated much haphazard light from outside the field is eliminated and the image is improved. This is an important point with high-power objectives. This scatter of light appears to be a more important limitation of thick tissues than mere insufficient illumination. Green and blue filters also reduce diffraction and, in some cases, improve contrast, but in practice they were found to help only rarely.

Another form of illumination tried in the early stages was a plastic Coldlite ophthalmic transilluminator with a built-in battery. As this has a cross-section of some 2 mm. at the tip, however, it proved satisfactory only for low-power work, and the curved tip proved rather bulky for use in small animals.

An attempt was made to reduce loss of light from the terminal part of the rod into the solution surrounding the tissue by silvering the terminal few millimetres. But unexpectedly this considerably reduced the intensity of the emerging light, probably owing to absorption by an inexpertly silvered surface.

The use of a much larger condenser of the twin plano-convex type should increase the illumination appreciably, but it is felt that the greatest room for improvement here is in the development of aspherical condensers, which should produce a much more accurate image of the light source. High-intensity discharge-tubes might prove to be useful sources of light.

The intensity of illumination was unfortunately not sufficient for the photography of moving tissues.

2. *Temperature Control*

A good account of temperature control is given by Knisely (1937).

Since most vascular beds are sensitive to variation in temperature, it is essential to maintain the tissue under examination at the physiological temperature. In theory this should be easy with a flow of warm solution bathing the tissue, but in practice an even temperature is difficult to achieve.

Two factors must be considered: (i) general cooling of the organ by exposure, and (ii) local heating by the source of illumination. Any exposure of the organ for visual examination must present a considerable cooling surface, particularly if it is a moist surface which can cool by evaporation as well as by conduction, and even if it is covered by a cover-slip, which is a poor substitute for layers of muscle, fat, skin and fur, the cooling

is appreciable. Heat must be supplied to counteract this cooling, but unless the source of heat be applied evenly between the cooling surface and the organ itself the correction is bound to be uneven and there will be a temperature gradient rising towards the source of heat. If the parts of the organ nearest to the source of heat be at the right temperature the more distant parts will be too cool, and if the temperature is raised to counteract this the nearer parts will be liable to overheating. By means of a flow of warm solution as the heat source, the rate of flow can be raised until the unevenness becomes negligible, but there is then a risk of setting up currents which move the organ and tend to stimulate the smaller vessels mechanically.

These temperature variations have been measured by means of a small thermocouple about 2 by 1 by 1 mm. in size. During the examination of the exposed mouse spleen, covered by a cover-slip, the temperature in the warm chamber formed beneath the cover-slip varied from one end of the spleen to the other by $1.5-2^{\circ}$ C. In practice, observations were confined to regions near the tip delivering the warm solution, and when regions further afield were examined the delivery tip was moved with the illuminating rod. No marked disturbances of the circulation from temperature differences were noted, but it is felt that such temperature differences are a potential source of error, which ideally should be eliminated. Knisely makes no mention of the range of temperatures observed at the surface of the organ. MacKenzie, Whipple and Wintersteiner, who completely exteriorized the animal's spleen, had a thermometer bulb placed near the spleen, but, as judged by thermocouple measurements, this could give only a rough guide to the average temperature within the chamber.

Local heating at the site of illumination is an even more difficult factor to eliminate. Such heating may be due either to conduction of heat down the rod, to heat-rays passing through the rod, or to the absorption of light-rays by the tissue and by their conversion to heat. The factor of conduction was unimportant here, as the source of light itself was not unduly hot; moreover, there was a filter of heat-absorbing glass which removed some 90 per cent. of the heating rays in front of the condenser, as well as a long air-gap between the filter and the rod. Heat-rays passing through the rod were also largely eliminated by the filter.

The factor of conversion of light to heat by absorption is not so easy to eliminate. Knisely and subsequent workers used a flow of solution through the terminal segment of the illuminating rod, which formed a changing layer of fluid between the tip and the tissue. This arrangement satisfactorily cools the layers of tissue nearest to the light, but, as absorption of light continues through the depth of the tissue, the deeper layers, and those uppermost which are under examination, can scarcely be affected by the cooling, unless the temperature of the solution be lowered to such a degree that the tissue nearest to the light becomes over-cooled.

With the present apparatus, no heating could in practice be detected with the thermocouple at the site of illumination, and no effects of heating were detected microscopically. If the intensity of illumination is kept at a minimum, and if the light is switched off whenever continuous observations are not being made, the tissue's own circulation is probably sufficient to disperse the little heat that is produced. Nevertheless, it should not be necessary to depend on the circulation to remove excess heat, as this imposes an unphysiological burden which may affect observations made on the smaller vessels, even though with this apparatus no ill effects were noticed.

Another source of temperature variability is in the rate of flow of solutions from the constant-temperature bath. Even through a relatively short length of thick-walled pressure tubing the heat loss is considerable, and the temperature of the solution emerging at the delivery tip varies appreciably with the rate of flow. It is necessary to decide upon a convenient rate of flow and to set the thermostat in the bath accordingly. Day-by-day variations appear to be negligible. The dependence of the temperature on the rate of flow is sometimes a nuisance, however, as it is often an advantage to be able quickly to change the rate of flow to suit animals of different sizes and to suit different operating techniques. The ideal would be to have the heating element and thermostat as close to the delivery tube as possible, but this presents technical difficulties which have not yet been solved.

3. *Mobility of the Apparatus*

The use of a freely movable arm to carry the microscope is a great advantage. In particular, rotation of the microscope through an axis at right angles to the optical axis is a great help in observing the free edges of organs—for example, liver and spleen—where the surface is rarely horizontal. If a normal microscope stand with the stage removed is used, a tilting animal table provides some degree of rotation, but on the whole this is less satisfactory, because the range of rotation is restricted and both the illuminating rod and the lamp have also to be readjusted. Moreover, a movable arm also allows the microscope to be swung clear of the animal while manipulations on the animal are being made, and to be swung back afterwards without risk of disturbing the preparation.

The illuminating rod should also be easily and smoothly movable in every plane, and provision should be made for its rotation in its own long axis, as well as in the horizontal axis at right angles to the long axis. The only movement which requires fine adjustment is that in the vertical plane, since the rod must be brought as close to the under-surface of the organ as is possible without exerting pressure. The height at which this is fulfilled is liable to vary slightly at different points of the organ.

A tilting animal table is nevertheless an advantage, even with a freely movable microscope, as diaphragmatic movements can be somewhat diminished by suitably tilting the animal. Horizontal adjustments of the table can be made satisfactorily enough by moving the base by hand.

OBSERVATIONS ON THE SPLENIC CIRCULATION

In the two recorded accounts of the splenic circulation as seen microscopically in the living animal there is a striking instance of the difficulty in interpreting circulatory patterns. Knisely (1936b) described the splenic circulation in mice, rats and cats as a system of rhythmically filling and emptying sinuses, which form, in the unstimulated spleen, the main connections between arterial and venous sides. He saw the arterioles opening by way of arterial capillaries into contractile cucumber-shaped vessels, with sphincters at either end. These either allowed the blood to pass in a continuous stream through them, or, closing their distal sphincters, filled up with blood from which the plasma filtered away through their thin walls, and thereupon entered a 'storage' phase, which lasted from a few minutes to 10 hours. At the end of this phase the sphincters opened and the closely packed red cells tumbled out along the short venous capillary into the collecting venule. A few capillaries, unbroken by these sinuses, directly connected

arteriole to collecting venule. In injured or dying spleens, on the other hand, red cells were seen to pass through the walls of the sinuses and invade the pulp-tissue (Knisely, 1936c). Only very occasionally were a few red cells seen to do this in unstimulated spleens. No striking differences between species were observed by Knisely and no mention is made of splenic contractions.

MacKenzie, Whipple and Wintersteiner (1940, 1941) sought to confirm Knisely's observations. They used his technique, with the sole difference that they exteriorized the spleen completely and housed it in a small chamber on the surface of the animal, whereas Knisely had merely allowed a portion of the spleen to present through an incision in the abdominal wall. The description of the circulation given by these authors is at variance in many respects with that of Knisely. The arterioles were seen to open by ampullae into the pulp interstices, and the red cells are described as travelling between the pulp-cells by haphazard channels towards the collecting venules, which they entered by existing stomata in the venule wall. For the most part these haphazard channels were fairly well defined, but they varied considerably in size, direction and number with the degree of splenic contraction. Random collections of red cells were seen lying among the pulp-cells, apparently stationary for some hours and not visibly confined in any way by a vessel wall.

These are, in essence, the two existing views on the splenic circulation derived from direct observation. Knisely supports a 'closed' circulation through intact, lined vessels, while MacKenzie and his associates contend that it is an 'open' circulation. The present study is an attempt to discriminate between these two opposing views.

MATERIAL AND METHODS

The animals used were 41 male mice of varying ages, and a few guinea-pigs. Observations on the guinea-pigs, however, are not included in the present findings, as they were too few for satisfactory conclusions to be drawn.

The mice were kept, fed and handled in the laboratory, so that splenic contraction from fright could be minimized. They were anaesthetized with Nembutal, 2-4 mgm. given subcutaneously over the hindquarters, further doses of 1 mgm. being given into the opened peritoneal cavity as required. The initial subcutaneous injection was given with a minimum of disturbance by gently holding the animal's tail while it gripped a wire mesh, and by picking up the loose skin over the hindquarters with a fine sharp needle on the syringe.

When anaesthetized the mouse was placed on its right side in a V-shaped trough cut in a thick slab of cork. This trough was grooved, and at the bottom was perforated to allow the Tyrode's solution to run away and to prevent the animal from lying in a pool of cold solution. The skin was sometimes shaved, but more often this was not done, as shaving contributed little and disturbed the animal unnecessarily. An incision was made in the skin of the left flank, extending downwards from the costal margin and at right angles to it. This incision was then extended at both ends, to form a skin flap which was either turned down or completely cut away. The abdominal musculature now exposed was cut parallel to the original skin incision and close to it, and the ventral tip of the spleen was extruded through this opening either by gentle manipulation of the abdomen or by levering it through with a non-metallic probe. The portion of the spleen thus extruded lay on the exposed abdominal musculature. While the spleen was being manoeuvred through the incision, the warm Tyrode was allowed to flow on to the exposed abdominal wall; as

soon as the spleen tip was exposed, a cover-slip was lowered on to its surface, where it remained in place by surface tension.

This procedure is similar to Knisely's. MacKenzie's technique of completely exteriorizing the spleen on a table placed on the abdominal wall was tried, but, in spite of reducing the respiratory movements of the spleen, it did not appear to help greatly and added considerably to the manipulation of the organ.

In this study periods of observation of longer than three hours were not attempted.

OBSERVATIONS

It may be worth recording some of the initial difficulties encountered. Respiratory movements, which impart to the spleen a vertical as well as a horizontal movement relative to the microscope, interrupt continuous observation of detail by a rhythmical loss of focus and by shift of the image. With the passage of time this becomes less important, and the separate images seen clearly at each stationary phase of respiration become subjectively fused. Slight pressure on the microscope arm in time with the respirations reduced the effect of vertical movements. Practice with the liver, in which the respiratory movements are much greater, made work with the spleen rather easier.

At first it was difficult to detect any vascular flow at all. The spleen appeared a mottled light-red colour, showing darker and lighter areas, but, beyond a slight granularity, no obvious details. The larger collecting veins were the first vessels to be detected; from these could be traced the smaller venules, and then slowly, after the first few trials, the irregular vascular pattern began to stand out. Difficulty in tracing details was experienced particularly when changing from a lower to a higher magnification, but the use of fine tips to the illuminating rods, and the consequent improvement in resolution, went far to alleviate this trouble. Nevertheless, it was necessary to learn to recognize the structures, and failure to do so could be an important source of subjective error in the method.

Examination of stained paraffin sections and unstained thick cleared sections confirmed that the mottled appearance which was the first striking feature of the transilluminated spleen, and most marked in those animals with small contracted spleens, was due to the relative amounts of red pulp lying over and between the lymphoid follicles.

The arterioles were seen as fine pink vessels with refractile walls emerging from the pale lymphoid tissue. The rate of blood-flow through them was too fast for individual red cells to be distinguished. These arterioles appeared to end in one of two ways. Usually the vessel disappeared in an indeterminate manner amongst the pulp-tissue; no indisputable vessels could be seen connecting with the end of the arteriole. Less often, the arteriole was continuous with one or more fine channels, sometimes again subdividing, but ultimately joining a venule after a relatively direct course.

The bulk of the red pulp consisted of three elements: the clear cells of the pulp 'cords,' aggregates of apparently stagnant red cells, and streams of blood of greatly varying size, direction and rate of flow. These streams of blood (the term 'streams' is used to avoid the assumption of any containing vessel) were most remarkable for their irregularity. They varied in size from about 8 to 40 μ in diameter, and from about 50 to several hundred μ in length. Some were tortuous and some straight, the smallest ones being, in general, the most tortuous. The direction of the flow was often different in adjacent vessels; numerous anastomotic channels were present, and the angles at which they joined one another varied from acute to obtuse. In spite of this irregularity,

however, in any group of these channels there was a general progression towards a collecting venule, and the smaller channels tended to open into the larger, although a medium-sized channel would often branch again into one small one and one of approximately its own size.

Most of these blood channels showed no definite limiting wall, but again appearances varied. The smaller the stream the more irregular were its boundaries, and in the smallest no semblance of an endothelium was ever seen, suggesting that here the red cells were merely travelling in procession between the naked pulp-cells. Some of these smallest streams appeared to be completely unbounded even by pulp-cells, and it was assumed that here the red cells were travelling in a relatively large volume of plasma; occasionally two or three of these 'free' streams could be seen emerging from the same point and travelling parallel and close together, but no definite point at which red cells were being dammed back or filtered off was ever seen. These 'free' streams joined other channels at apparently fixed open junctions. A few of the small and medium-sized channels, 2-5 red-cell diameters wide, did show definite refractile smooth walls over a short length of their course, but this was not necessarily carried on beyond the next junction. The collecting venules all had smooth and just perceptible walls. The entry of these blood-streams into the collecting venules appeared to be by way of definite junctions and not by mere stigmata in the wall of the venule.

In none of the animals examined were any vessels seen which acted as reservoirs for the red blood-cells. No definite sphincters were seen, although there were occasional fixed constrictions, sometimes at the junction of one blood channel with another, and sometimes along the middle of the course of a channel. The rate of flow behind such constrictions might be slow, but no progressive damming back of blood was seen.

No obstruction to blood-flow by leucocytes, as described by MacKenzie *et al.* (1941), was seen.

In general, there was no marked or constant variation in the rate of flow along the channels, but certain types of variation were nevertheless observed. The flow in a small anastomotic channel occasionally became sluggish and then ceased, the channel remaining either full or empty of blood. This may be related to splenic contraction, though the rhythmical contractions in the mouse are too small and unobtrusive to allow one to be certain. In some of the larger vessels, when the flow was sluggish, there might be seen a slight oscillation of the blood with the respiratory movements. In many of the channels, an irregular slowing of the flow might occasionally be observed, but this appeared to have no effect on the flow in that group of channels as a whole.

On one occasion only a rhythmical variation in flow was noticed. In one of the larger channels, which in this case ran nearly across the low-power field of the microscope and appeared to connect two networks of smaller streams, the flow ran fast for about 25 seconds and then slowed down or even reversed for 5-10 seconds. Over the course of five minutes the rhythm was broken only once, when the fast flow was maintained for 82 seconds. The actual figures for the periods of fast flow were consecutively 25, 28, 25, 20, 20, 25, 82, 16 seconds, and for slow flow 7, 5, 10, 8, 9, 15 seconds. No explanation for this was found, and the phenomenon was not seen again.

Another type of variation was seen where an arteriole terminated directly in a blood channel. At a bifurcation of this channel, blood could be seen travelling first along one branch, then along both, then along one or the other again. There was no obvious rhythm

to this variation, and the flow did not necessarily cease entirely in the temporarily inactive branch. Again, no cause for this variation was seen either at the site or distal to it.

It yet remains to describe the mode of connection of the arterioles to the numerous ramifying blood-streams. All that can be said at present is that these blood-streams originate very close to the ends of the arterioles, and that a few can be seen to communicate directly. The ampullae described by MacKenzie, Whipple and Wintersteiner (1941) at the ends of the arterioles seem to be a close approximation to the truth, but what becomes of the arteriolar endothelium has not yet been seen. Knowledge of this should contribute to a more exact picture.

The scattered and apparently stagnant red cells which occurred throughout the red pulp remain enigmatic. They occurred singly and in irregular aggregates of all sizes. Although some were watched for periods of two hours and more, no migration and no diapedesis through the boundaries of the channels could be detected. Owing to the lack of clear differentiation of cell boundaries in unstained living tissue, the exact relation of the red cells to the pulp-cells, and the nature of the obstruction to free movement, remained undiscovered.

DISCUSSION

The existence of numerous, tortuous, ramifying, anastomosing and irregularly bounded small blood-streams, without detectable endothelial walls, points to an open type of circulation through the splenic pulp, in the sense that the blood appears to be in intimate contact with the pulp-cells. These observations are entirely in accord with those of MacKenzie and his associates. No definite conclusions can, however, be drawn as to the presence or absence of a lining membrane to these channels, and it can only be said that they have not been seen in the present series. The relative constancy of the vascular pattern—perhaps most noticeable in the mouse, where the splenic contractions are negligible—suggests that there may be preformed channels lined by a thin endothelium. Reticulum studies by Foot (1927*a*, 1927*b*) and by Snook (1944), on human and guinea-pig spleens respectively, suggest that this is the case. The presence of a large proportion of the blood lying among the pulp-cells outside the obviously moving blood-streams in an undamaged organ is, however, strong evidence for an open circulation, and McNee (1931) remarks on this feature of the mouse spleen. Nothing was seen resembling the closed circulation of the type described by Knisely (1936*b*), with its rhythmically filling and emptying sinuses, and, although according to this author (1936*c*) stimulation of the spleen by cutting, scratching, bruising or squeezing led to a rapid outpouring of red cells into the pulp interstices, it was certain that in the present series any such gross stimulation was avoided. Further, no such marked deviations from the normal were noted in spleens which had been roughly handled.

Many problems still remain unsolved. Do the arterioles in fact pour their contents among the pulp-cells, to let the blood find its own path between them? How do stagnant red cells come to lie still within the pulp? What relation have they to the pulp-cells? What is their ultimate fate? Do they differ from the red cells which cross the pulp-tissue via the sinuses? To what extent does the circulatory pattern differ in different species? In the cat, for example, splenic contractions are prominent and ellipsoids are present in the arterioles; in the mouse contractions are feeble and ellipsoids absent. These problems appear to be only half answered, and such answers as there are have not always been confirmed.

The present series of observations must be regarded as very incomplete, but they are enough to suggest that to provide a clear solution any single technique is inadequate. Microscopic appearances in the living organ must be checked and confirmed by routine histological methods, by the use of thick sections, of special stains, such as reticulum and red-cell stains, of injection and perfusion techniques on the living and isolated organ, and by the use of radiography of the vascular pattern by the injection of radio-opaque substances (Trueta *et al.*, 1947). Any observations made should be confirmed, as far as possible, on other species, in particular on the primates and man.

SUMMARY

1. Previous methods used for the illumination of living tissues are briefly reviewed.
2. A technique is described which aims at visual observation of circulatory phenomena under physiological conditions. Anaesthesia, exposure of the tissue, and replacement of coelomic and interstitial fluids by artificial solutions represent departures from the physiological which it has not been possible to avoid. Temperature control, achieved most easily by a flow of warm physiological solution bathing the tissue, presents certain problems, which are discussed.
3. The tissue examined was illuminated by a 24-watt filament source focused on the end of a glass rod, the light being conducted along this rod by internal reflection to the area under the microscope. The details of the apparatus are discussed and are summarized diagrammatically.
4. Some observations on the circulation of the living mouse spleen are described; they suggest that the flow of blood through the red pulp is by haphazard channels between the splenic cells, these channels remaining fairly constant in the absence of extrinsic stimulation of the spleen. No rhythmical intermittent activity of these blood channels was observed.

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SOME SPECIES OF *CULICOIDES* (DIPTERA, CERATOPOGONIDAE) FROM THE STATE OF CHIAPAS, MEXICO

BY

J. W. S. MACFIE

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I have been privileged to examine a portion of a large collection of Ceratopogonidae (estimated to contain some 20,000 specimens) from the State of Chiapas, Mexico, sent to the Commonwealth Institute of Entomology by Dr. Alfonso Dampf. The greater part of the collection was made by Dr. Dampf himself from May to July, 1935, during a visit to study and investigate onchocerciasis. His headquarters then was the coffee plantation El Vergel on the right slope of the Huixtla valley, at an altitude of about 700 m. and some 20 km. distant from the Huixtla railway station. Here, about 200 m. from the river Huixtla—a mountain brook with cascades and rapids—the slopes are covered by virgin forest, and the plantations are situated in small clearings which still retain their original inga trees for shade.

The visit was paid during the rainy season (which begins in May), and Dr. Dampf notes that *Culicoides* were 'the worst pest, sometimes unbearable.' So numerous were they, and so vicious, he says, that their attacks were likened to sand thrown against the face. They had, too, the objectionable habits of disappearing into the hair of the head to bite the scalp, and of entering any opening to be found in the clothing. They were most troublesome near pasture-land. Subsequently, additional captures of Ceratopogonidae were made in this region by Mr. José Parra, an inspector in the Onchocerca organization, during his tours through the affected zone in August, 1935, and by Dr. Roberto Nettel, the medical chief of the Onchocerca brigades, in June, 1938. But, unless otherwise stated, all the specimens referred to hereafter were collected by Dr. Dampf, and were taken at night by means of a trap-lamp.

The portion of this collection examined by me includes 3,335 specimens of *Culicoides*. Of these more than 97 per cent. belong to the species I refer to here as *guttatus* (Coq.). It was in specimens of this species that developing filariae were found by Dr. Dampf (1936). The small number of specimens referable to other species include examples of *debilipalpis* Lutz, *insignis* Lutz, *loughnani* var. *jamaicensis* Edw., *luteovenus* Root and Hoffman, and *pusillus* Lutz, and representatives of 10 other species which I believe to be new and which therefore are described below. The method adopted in the descriptions is that which I have employed in a number of recent reports. The unit used is approximately 3.7μ . The figures of the wings, although drawn with the aid of a camera lucida, are semi-diagrammatic and are intended only as illustrations of the adornment. The macrotrichia often are not shown.

It is customary when reporting on collections of Ceratopogonidae to approach them from a regional standpoint; indeed, there is often no alternative. Fox (1946) has recently upheld this procedure in the case of Caribbean species of *Culicoides*, and has pointed out that 'it is inadvisable to synonymize species whose type localities are widely separated,

particularly if the evidence for the synonymy is to be found in only one sex.' He is undoubtedly correct. But it is also true that many species have an extensive range of geographical distribution, and, I think, the possibilities of drift and of dispersion by other means of such small insects should be recognized. In dealing with Dr. Dampf's collection from the State of Chiapas, therefore, it seemed to me illogical not to take into account species known to occur in neighbouring parts of Mexico, even if the physical features of the areas are very different. In the key which follows, therefore, for better or for worse I have included with species from the Caribbean region others known to occur in regions ranging from southern Mexico to Brazil.

In drawing up this key I have relied almost entirely on wing characters. That is natural, because in the genus *Culicoides* it is in the adornment of the wings that specific characters are most commonly found. I have not been able to follow Hoffman by making any considerable use of thoracic adornment. In some species the presence of small spots on the thorax or of a conspicuous pattern composed of dark and light patches may be of much assistance, but the minutiae of thoracic adornment I find too variable and too much dependent on the condition of the individual specimen to be serviceable. So, too, I have been unable to make use of the apparent obsolescence of veins or parts of veins as Fox (1946) has done.

I have thus taken into account more than 60 species, but I cannot hope to have included all those that should have been considered, because the relevant literature is both scattered and extensive. As I myself have had the opportunity of examining authenticated examples of very few of the species, and as I have therefore had to rely mainly on the descriptions and figures of others, it is inevitable that errors have been made. It is for this reason that I have often given in the couplets of the key supplementary characters in addition to the main alternatives. If, by the publication of this key, the distribution of the species of *Culicoides* found in this region is brought to the critical notice of those with direct personal knowledge of them, I think it will have served a useful purpose.

The key, which is designed to show how the females may be distinguished, is applicable also to such males as are known if it is borne in mind that in them the pale markings on the wings are more diffuse and the macrotrichia less abundant than they are in the females. The identification and study of more of the males is, indeed, a pressing need not only to confirm or disprove the rather fine distinctions drawn between some of the females, but also to support or annul some suggested synonymies, and because, as shown by Root and Hoffman (1937), a study of their terminalia may well lead to a rational grouping of the species.

From this key *pictipennis* (Philippi), a Chilean species described in 1865, has been omitted. It would run down to *guyanensis*, and it may prove, of course, that this species has so wide a geographical range that it extends to Chile, but even if it does so Philippi's name will not be valid for it, because it is preoccupied by Staeger's. Other species which find no place in the key for one reason or another include four of Williston's (1896) species from St. Vincent (West Indies) which have been believed to be *Culicoides*. Thanks to the courtesy of Mr. Paul Freeman of the British Museum I have been enabled to examine paratypes of three of them, namely, *pygmaeus* which is a *Dasyhelea*, *laturatus* which is an *Atrichopogon*, and *lotus* which is a *Forcipomyia*. The fourth, *decor*, is, however, a *Culicoides*. Mr. Freeman has most kindly examined the type, in which, he tells me, the 'wing markings are faint except for the three markings along the costa, and they appear to form three

2. Second radial cell more or less covered by pale area ... 3
 Second radial cell entirely covered by dark area ... 11
3. Pale zone in fork of vein Cu and along at least parts of Cu 1 and Cu 2 ... 4
 No such pale zones ... 7
4. Pale spot straddling middle of vein M 2 ... 5
 No such pale spot ... *oliveri* Fox
5. Pale spot enveloping cross-vein quite narrow, covering less than half first radial cell. Conspicuous pale spot straddling base of vein M 1. Only a single pale spot in distal part of anal cell. Large species ... *heliconiae* Fox
 Pale spot enveloping cross-vein larger, covering half or more of first radial cell ... 6
6. Two pale spots in cell R 5 narrowly separated. Cross-vein often infuscated. Usually two pale spots in cell M 1 distal to the pale spot straddling vein M 2, but most distal one (at margin of wing) may be almost or quite obsolete, especially in males ... *guttatus* (Coq.)
 Syn. *diabolicus* Hoff., *filariferus* Hoff., and (?) *bimaculatus* Floch and Abonnenc, *painteri* Fox, *pseudodiabolicus* Fox, and *trinidadensis* Hoff.
 Two pale spots in cell R 5 more widely separated. Only a single pale spot in cell M 1 distal to pale spot straddling vein M 2 ... *insignis* Lutz
 and (?) syn. *inamollae* Fox
7. Wings pale, pale with dark markings rather than the reverse. Distal pale spot in cell R 5 large, occupying almost whole tip of wing anterior to distal part of vein M 1. Dark spots in cells M 1 and M 2 conspicuous, but no dark spot in middle of cell Cu 1 ... *elutus* sp. nov.
 Wings darker, grey with pale markings. Distal pale spot in cell R 5 smaller, set back from tip of wing ... 8
8. Pale spot present immediately anterior to fork of vein Cu, and another just above base of vein M 1 posterior to interspace between radial cells ... 9
 No pale spots in these situations. Pale spot enveloping cross-vein continued posteriorly as a band well into anal cell ... *luteovenus* R. and H.
9. Pale spot near periphery in cell M 2 small, not reaching to margin of wing. Third palpal segment slightly inflated, with shallow subdivided pit ... *hylas* M.
 Pale spot near periphery in cell M 2 larger, reaching to margin of wing. Palpi slender, third segment without pit ... 10
10. Pale spot near middle of cell R 5 large, reaching to margin of wing anteriorly. Spermathecae with short portion of commencement of duct chitinized ... *verecundus* sp. nov.
 Pale spot near middle of cell R 5 quite small, reaching neither to wing margin nor to fold above vein M 1. Spermathecae with no part of duct chitinized ... *palpalis* sp. nov.
11. Pale spots at periphery of wing very ill defined ... 12
 Pale spots at periphery of wing well defined. Macrotrichia more or less abundant ... 14
12. Almost no macrotrichia ... *pusillus* Lutz
 Macrotrichia fairly numerous on distal half of wing ... 13

13. Third palpal segment slightly swollen, with pit in apical half ... *bambusicola* Lutz
Third palpal segment without pit ... *marium* Lutz
14. Cell R 5 with pale spot situated close to tip of wing ... 15
Cell R 5 with most distal pale spot set well back from tip of wing 20
15. Pale spot in cell R 5 situated close to tip of wing large. Mesonotum
not spotted ... 16
Pale spot in cell R 5 situated close to tip of wing small or very
small ... 17
16. Pale spot in cell Cu 1 reaching to margin of wing, but not to
either veins Cu 1 or Cu 2. Spermatheca single, with long duct
part ... *crepuscularis* Mall.
Pale spot in cell Cu 1 in contact with vein Cu 1. Spermathecae
two, with short duct part ... *albomaculata* R. and H.*
17. Only two widely separated pale spots in cell R 5 distal to level of
end of costa. Only a single pale spot in distal half of cell M 1.
Mesonotum not spotted ... *haematopotus* Mall.
Three pale spots in cell R 5 distal to level of end of costa ... 18
18. Two pale spots in distal half of cell M 1. Mesonotum spotted ... 19
Only a single pale spot in distal half of cell M 1. Mesonotum
not spotted but conspicuously adorned with dark and yellowish
patches ... *pampoikilus* sp. nov.
19. Pale area present immediately posterior to radial cells, but none
in fork of vein Cu ... *arubae* Fox
No pale area immediately posterior to radial cells. Pale area in
fork of vein Cu and bordering parts of Cu 1 and Cu 2 ... *variipennis* (Coq.)
20. With a pale spot straddling middle of vein M 2 ... 21
No pale spot in this situation, straddling vein M 2 ... 32
21. Fourth tarsal segments cordiform ... *amazonius* M.
Fourth tarsal segments subcylindrical, not cordiform ... 22
22. Pale spot straddling basal part of vein M 1 distinct, divided
between cells R 5 and M 1 ... 23
This pale spot absent, indistinct, or reduced to a small pale area
in cell R 5 only ... 26
23. Cross-vein infuscated, forming dark spot in middle of large pale
area. Veins not pale-margined. Large species; length of wing
about 1.5 mm. ... 24
Cross-vein not infuscated, and pale area enveloping it smaller.
Veins pale-margined at ends at any rate. Smaller species;
length of wing about 1.2 mm. ... 25
24. Pale spot straddling basal part of vein M 1 confluent with pale
spot at end of costa. Distal pale spot in cell R 5 large, dark
band between it and pale spot at end of costa very narrow ... *dampfi* R. and H.
Pale spot straddling basal part of vein M 1 not confluent with pale
spot at end of costa. Distal pale spot in cell R 5 much smaller,
and separated from pale spot at end of costa by broader dark
band ... *scopus* R. and H.

* Johannsen's (1943) version of this name is *albomaculatus*, perhaps correctly.

25. Darkened area on basal half of costa narrow. Veins M 1, M 2, Cu 1 and Cu 2 all pale-margined *loughnani* Edw.
 Darkened area on basal half of costa long, extending practically to base of wing. Veins not pale-margined except (M 1, M 2 and Cu 1) at tips *loughnani* var. *jamaicensis* Edw.
26. A single pale spot in distal part of anal cell 27
 Two pale spots in distal part of anal cell either quite separate or more or less fused to form an hourglass-shaped mark 29
27. Pale area occupying fork of vein Cu and bordering also parts of Cu 1 and Cu 2. Distal pale spot in cell R 5 double. Mesonotum conspicuously adorned with dark and lighter patches *baueri* R. and H.
 No pale area in fork of vein Cu or bordering Cu 1 and Cu 2. Distal pale spot in cell R 5 not double. Mesonotum not conspicuously adorned 28
28. Macrotrichia scanty, limited to tip of wing *phlebotomus* (Will.)
 Macrotrichia more abundant, covering most of wing, and numerous in anal cell. Spermathecae two, retort-shaped *alambiculorum* sp. nov.
29. Pale spot enveloping cross-vein large, reaching posteriorly to about midway between veins M and Cu. Mesonotum not conspicuously adorned (♂) *propinquus* sp. nov.
 This pale spot smaller, extending only just beyond vein M. Mesonotum conspicuously adorned. Spermathecae pyriform or obovate 30
30. Distal pale spot in cell M 1 reaching to margin of wing *pampoikilus* sp. nov.*
 Distal pale spot in cell M 1 set back from margin of wing 31
31. Distal pale spot in cell R 5 set close to pale spot just beyond end of costa, nearer to it than to tip of wing *poikilonotus* sp. nov.
 Distal pale spot in cell R 5 separated more widely from pale spot at end of costa, about midway between it and tip of wing (♂) *daedalus* sp. nov.
32. A single small pale spot in cell M 1 remote from margin of wing. Mesonotal adornment of three longitudinal dark lines *trilineatus* Fox†
 Three pale spots in cell M 1 33
 Two pale spots in cell M 1 34
33. In distal part of cell R 5 (i.e., in the part beyond the pale spot at end of costa) a group of three pale spots arranged in a triangle *guyanensis* Floch and Abonnenc
 Syn. *recifensis* Barbosa, and *stibalensis* Fox
 Two pale spots in this area, one near middle of cell R 5, the other at tip just above end of vein M 1 *paraensis* (Goeldi)
 Syn. (?) *undecimpunctatus* Kieffer
 Two small pale spots in this area, the one anterior to the other, both well away from tip of wing *propriipennis* sp. nov.
34. Two pale areas in cell R 5 distal to level of end of costa, with small pale spot between them which may be joined on to distal spot. Most distal pale spot set back from tip of wing, not just above end of vein M 1 35
 No such small spot between the larger pale spots in cell R 5 38

* This species is entered here a second time (see couplet 18) because the small pale spot just anterior to the tip of vein M 1 is rather indistinct and might be overlooked.

† According to Fox (1946) cell M 1 'with one or two light spots.' Should there be two, this species would seem to be very near *C. debilipalpis* Lutz.

35. Small spot in cell R 5 between the two larger pale areas situated just above vein M 1, between it and the fold anterior to it. Mesonotum not spotted, but adorned with dark and paler patches ... 36
This small pale spot situated more anteriorly, above fold anterior to vein M 1. Macrotrichia more or less abundant, not limited to cells R 5 and M 1 ... 37
36. Pale areas in cell R 5 just beyond end of costa, composing a group of three small spots. Distal pale spot in cell R 5 about midway between this group and tip of wing. Macrotrichia scanty, in cells R 5 and M 1 only ... *castillae* Fox
Distal pale spot in cell R 5 situated much nearer to pale areas at end of costa than to tip of wing. Macrotrichia more abundant, covering distal half of wing and present also in cell Cu 1 and in anal cell ... *cacozelus* sp. nov.
37. Pale spot in cell R 5 just beyond end of costa small, not connected with pale area immediately posterior to radial cells. Mesonotum not spotted but conspicuously adorned with pattern of rather large patches ... *reticulatus* Lutz
Pale spot in cell R 5 just beyond end of costa more or less confluent with pale area immediately posterior to radial cells. Mesonotum spotted ... *furens* (Poey)
Syn. *maculithorax* (Will.)
38. Two distinct pale spots in cell M 2 distal to level of fork of vein M 39
Only one pale spot in this region ... 42
39. Macrotrichia scanty, not extending towards base beyond level of end of costa. Two spermathecae ... 40
Macrotrichia more numerous, extending in line to base between M and Cu. One spermatheca ... 41
40. Macrotrichia in cell R 5 only. Pale spot at periphery in cell M 1 reaching margin of wing; that in cell Cu 1 reaching both to margin of wing and to vein Cu 1 ... *wokei* Fox
Macrotrichia rather more numerous, a few in cells M 1 and M 2 as well as in cell R 5. Pale spots near periphery in cells M 1 and Cu 1 not reaching to margin of wing. Two spermathecae. Eyes bare ... *fluvialis* M.
41. Pale spot at end of costa hourglass-shaped. No pale spot immediately anterior to fork of vein Cu. Eyes bare ... *pulchripennis* M.
Pale spot at end of costa divided into two small, separate, spots. Pale spot present immediately anterior to fork of vein Cu. Eyes hairy ... *eublepharus* sp. nov.
42. Macrotrichia extending to base between veins M and Cu. Third palpal segment with very deep pit. Antennae with flagellum segments forming almost continuous series, 10 and 11 nearly same length. Two spermathecae ... *debilipalpis* Lutz
and (? syn.) *borinqueni* Fox
Macrotrichia more scanty, few if any nearer to base than level of end of costa ... 43
43. Pale spot about middle of cell R 5 oval or reniform, as in *debilipalpis*. Eyes bare. Palpi, antennae and spermathecae as in *debilipalpis* ... *debilipalpis* var. *glabrior* M.
Pale spot about middle of cell R 5 rounded ... 44

44. Pale spot present immediately anterior to fork of vein M, and fork of Cu enveloped in a pale area. Cross-vein dark. No palpal pit *acotylus* Lutz
Syn. *panamericanus* Fox
- No such pale markings, and cross-vein not dark. Palpal pit well developed 45
45. Two small pale spots just beyond end of costa, the one lying anterior to the other 46
- The more posterior pale spot situated near end of costa separate, and lying posterior to second radial cell, much as in *debilipalpis*. Eyes hairy 47
46. Pale spot present immediately anterior to fork of vein Cu. Pale spot at periphery in cell M 2 larger than corresponding spot in cell M 1 *hoffmani* Fox
- No pale spot immediately anterior to fork of vein Cu. Pale spot at periphery in cell M 2 smaller than corresponding spot in cell M 1 *horticola* Lutz
47. Antennae with flagellum segments forming an almost continuous series, as in *debilipalpis*. Two spermathecae *germanus* M.
- Antennae with abrupt change of shape between segments 10 and 11. One spermatheca *dasyophrus* M.

Culicoides guttatus (Coq.)

El Vergel, V-VII 1935, 52 ♂, 2,378 ♀; mountain ridge between Rio Huixtla and Rio Des poblado, 1,000 m., in virgin forest, 3 VI 1935, 1 ♂, 71 ♀; Finca Victoria, VI 1935, 10 ♂, 259 ♀; Huixtla railway station, 4 VII 1935, 2 ♂, 6 ♀, at light in hotel in evening; Finca Palenque, 1,000 m., 10 VIII 1935, 62 ♀ (L. Grajales); Las Cabañas, Indian settlement, 1,170 m., 11 VIII 1935, 43 ♀, 'captured on the wall, attracted by light' (J. Parra); El Carrizal, 13 VIII 1935, 11 ♀ (J. Parra); Pacayal, 20 VIII 1935, 4 ♂, 44 ♀ (J. Parra); Nuevo Amatenango, 920 m., 23 VIII 1935, 1 ♂, 37 ♀ (J. Parra); Belisario Dominguez, Huixtla valley, 680 m., 24 VIII 1935, 42 ♀ (J. Parra); coffee plantation, Lubeca, near San Cristobal, 950 m., 11 III 1938, 1 ♀, 'swept over boulders in a brook'; and Finca Esperanza, 17-29 VI 1938, 22 ♂, 204 ♀, at light (R. Nettel).

All these insects are, I believe, *C. guttatus* (Coq.). They are no doubt the same as those taken also by Dr. Dampf at the same place in June, 1935, for which Hoffman (1939) proposed the name *C. filariferus*. Those specimens had been preserved in alcohol and may well have been somewhat discoloured when examined by Hoffman, and this, or the effects of the toluol technique to which he subjected them (which may also have altered their natural appearance), may account for his describing them as a new species. The species is, according to Hoffman, 'closely related to *C. venustus*,' and has 'affinities with *C. guttatus* and *C. diabolicus*.' *C. venustus* is peculiar, it seems, in having the radial cells 'separated by a distance equalling the length of either'; and in males, although the hypopygium is very similar, the small hairs on the claspers extend over more than two-thirds of their lengths, instead of being limited to the basal halves, as they are in *C. diabolicus*. But it is not clear how the other three species may be separated, and I should be inclined to regard them as conspecific. Vargas (1944) considers *filariferus* to be a synonym of *diabolicus*.

As I have noted elsewhere (1937), the species is liable to vary considerably. The examination of the large number of specimens in this collection has confirmed my previous observations. In these Mexican specimens the infuscation on the cross-vein is usually inconspicuous, and the two pale spots on the wings in the distal part of cell M 1, although generally distinct and separate, are in some individuals partially, in others completely, fused so as to form a single, often hourglass-shaped, spot.

***Culicoides insignis* Lutz**

Nuevo Amatenango, 920 m., 23 VIII 1935, 1 ♀ (J. Parra); and San Geronimo (now Belisario Dominguez), Indian village in the Huixtla valley, 680 m., 11 III 1938, 1 intersex form (antenna of female, hypopygium of male), 'trap-lamp at river, facing rocky mountain slope with virgin forest.'

In these specimens the most distal pale spot which is present in cell M 1 of the wing of *C. guttatus* is completely lacking. They should perhaps be regarded as examples of *C. insignis*, if that species is in fact different from *C. guttatus*. In the female the only difference which I have detected is that the pale spot near the middle of cell R 5 is separated from the pale area at the end of the costa rather more widely than it is in *C. guttatus*. In the intersex specimen the cross-vein is more deeply infuscated than it is in most of the specimens of *C. guttatus* taken in this locality. The hypopygium very closely resembles that of *C. guttatus*, but differs in one respect—the tips of the harpes are filiform and are without small terminal branches. This difference, if it is constant in normal males, may be a useful diagnostic character.

With regard to *C. insignis*, it may be said here that da Costa Lima (1937) has pointed out that the name should be applied also to the species figured by Lutz (1913) as *C. guttatus*, and to the specimen from Colombia which in 1932 (on the strength of Lutz's figure) I also attributed to that species. Da Costa Lima considers it probable that *C. trinidadensis* Hoff. is a synonym of *C. insignis*. To judge by Hoffman's figure (1925) of the wing of his species, there may be, however, a slight difference between the two species, the two pale spots in cell R 5 being in *C. trinidadensis* only narrowly separated, and so situated much more closely together than they are in Lutz's figure of the wing of *C. insignis*. They are in fact more nearly as in *C. guttatus*, with which species *C. trinidadensis* may indeed be conspecific, as I have elsewhere (1937) suggested.

***Culicoides elutus* sp. nov.**

A dark-brown species of medium size with extensive pale markings on the wings, resembling the North American species *C. cockerelli* (Coq.) in many respects, but differing as indicated below.

FEMALE. Length of wing 1.6–1.8 mm., greatest breadth 0.6–0.7 mm.

Head very dark brown. Eyes bare, contiguous above. Palpi darkish brown, slightly longer than proboscis; third segment somewhat inflated, with rather large, shallow, pit in distal half; lengths of last three segments in one specimen about 26, 9 and 11 units respectively. Antennae missing.

Thorax very dark brown, not conspicuously adorned and not spotted. Scutellum very dark brown; bearing four bristles and a few (eight) small hairs.

Wings with extensive pale markings, so that the general appearance is rather that of a pale wing with dark markings than the reverse. Adornment as shown in fig. 1. Cell R 5 with pale spot covering greater part of second radial cell; beyond it a dark hourglass-shaped area, and then a pale area which occupies whole tip of wing anterior to vein M 1. A conspicuous dark spot present in distal part of cell M 1 and another in cell M 2, but no dark spot in middle of cell Cu 1. Fork of vein Cu in dark area. Dark area along vein Cu 2 separating pale spot in cell Cu 1 from that in anal cell narrow. Two pale spots in distal part of anal cell, which, however, are narrowly connected. Costa somewhat thickened just above junction of radial cells. Macrotrichia abundant on distal half of wing, reaching almost to base between veins M and Cu, and numerous in anal cell. Halteres with white knobs.

Legs almost uniformly dark brown, but knees paler, yellowish. T.R. about two. Fourth tarsal segments subcylindrical, not cordiform.

Abdomen dark brown, but not so dark as mesonotum. Spermathecae two, well chitinized, obovate, subequal, total length and greatest breadth about 67μ and 52μ respectively. Rudimentary third spermatheca and ring present.

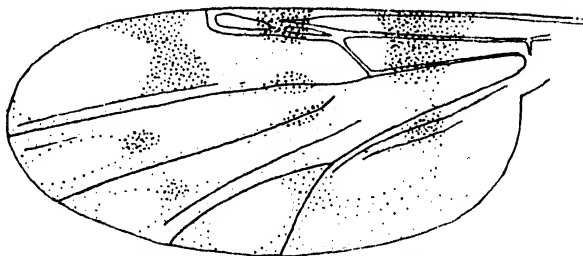


FIG. 1. *Culicoides elutus* sp. nov. Wing of female.

El Carrizal, 1,000 m., 13 VIII 1935, 2 ♀, 'at light' (J. Parra).

This species, one of the *pulicaris* group, resembles closely, it seems, *C. cockerelli* (Coq.). It may be distinguished from it, however, by the following points, according to the most recent and most detailed description of *C. cockerelli*—that of Hoffman (1925). It is a somewhat smaller species, the length of the wing being less, instead of rather more, than 2 mm. The eyes are contiguous above, as they are in most species of the *pulicaris* group, not 'rather widely' separated (cf. *C. griseus* Edw.). And in the adornment of the wings, to judge by Hoffman's figure, the distal pale spot in cell R 5 is larger, occupying more completely the tip of the wing, and the dark area covering the vein Cu 2 is narrower. It may be hoped that males, when available, will furnish additional characters to clarify the relationship of this species to *C. cockerelli* (Coq.), its variety *tristriatus* Hoff., and other species of the *pulicaris* group.

***Culicoides luteovenus* Root and Hoffman**

El Carrizal, 1,000 m., 13 VIII 1935, 4 ♂, 39 ♀ (J. Parra).

***Culicoides verecundus* sp. nov.**

A dark-brown species of medium size, resembling in many respects *C. indianus* Macfie, but differing apparently as indicated below.

MALE AND FEMALE. Length of wing 1.3–1.6 mm., greatest breadth 0.4–0.6 mm.

Head very dark brown. Eyes bare, so far as can be seen without macerating the specimens. Palpi very dark brown, slender, as long as proboscis; lengths of last three segments about 40, 17 and 13 units respectively in female, and 20, 11 and 9 units in male; third segment subcylindrical, not inflated, without definite pit, but bearing sensory hairs scattered over middle third. Antennae with torus very dark brown. In female, basal segments half brown, half yellowish; segments 4–10 subequal, about 15 by 8 units; 11–14 also subequal, 20–25 by 7–8 units; 15 longer, about 38 units, tapering distally. In male, antennae of usual form.

Thorax very dark brown above, mottled with large paler-brown areas. Scutellum very dark brown, bearing the usual 3–4 bristles and a few small hairs.

Wings (fig. 2) with adornment somewhat similar to that of *C. indianus* Macfie. The distal pale spot in cell R 5 extends anteriorly to the margin of wing, however; the peripheral pale spot in cell M 2 is larger than that in cell M 1, and not vice versa; there is

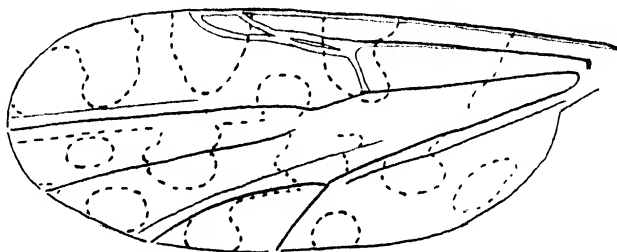


FIG. 2. *Culicoides verecundus* sp. nov. Wing of female.

a rather indistinct pale area overlying base of vein M 1 at level of interspace between radial cells; and distal part of vein M 1 is more or less distinctly bordered with a pale zone. Cross-vein not infuscated. Macrotrichia in female numerous in cell R 5, reaching to level of end of first radial cell, restricted to tip in cells M 1 and M 2, and only a very few in cell Cu 1; in male, more scanty, almost all of them in cell R 5.

Legs rather dark brown. Tibiae of four anterior legs with pale sub-basal bands only, those of hind pair with apical pale zones in addition. Form of segments normal; T.R. in both sexes about two, fourth segment of tarsi subcylindrical. Claws normal.

Abdomen about as dark brown as thorax. Spermathecae two, well chitinized, sub-spherical, unequal, diameters about 40μ and 50μ respectively, the duct narrow and chitinized at its commencement for only a very short distance, about 3μ . Rudimentary third spermatheca and ring present. Hypopygium dark brown, but claspers paler, yellowish; resembling that of *C. austeni* C., I. and M. Ninth segment with tergite cleft, square-ended, without apico-lateral processes, but with small conical projection in middle line posteriorly; sternite somewhat excavated in middle line. Side-pieces dark brown, with almost straight root-like process to articulate with harpe. Claspers pale, yellowish, bearing microtrichia on basal third only. Harpes fused basally, tapering rapidly to finely filiform ends. Aedeagus as in *C. austeni* or *C. venustus* Hoff.

El Vergel, 8 VI 1935, 1 ♂, 1 ♀, taken by means of a trap-lamp in forest, 7 p.m. to morning, together with numerous specimens of *C. guttatus* (Coq.); and Belisario Dominguez, Huixtla valley, 680 m., 24 VIII 1935, 1 ♀ (J. Parra).

These insects are probably the same as the single female from British Guiana resembling *C. indianus* Macfie to which I referred in 1940; if I now suggest for them a separate specific name it is on account of geographical separation and because the male of *C. indianus* is still unknown. From other species found in the Caribbean region which have somewhat similar adornment of the wings, they differ, according to Fox's recent review (1946), and, to select only a single character, in having no pale area in the fork of vein Cu.

***Culicoides palpalis* sp. nov.**

A dark-brown species of medium size, resembling the species described here as *C. verecundus* sp. nov., but with the pale wing-spot situated near the middle of cell R 5 smaller, and with no part of the duct of the spermathecae chitinized.

FEMALE. Length of wing 1.5–1.6 mm., greatest breadth 0.6 mm.

Head very dark brown or blackish. Eyes bare, so far as can be seen without macerating the specimen. Palpi (fig. 3A) very dark brown, long and slender, as in *C. verecundus*; lengths of last three segments about 42, 19 and 13 units respectively; third

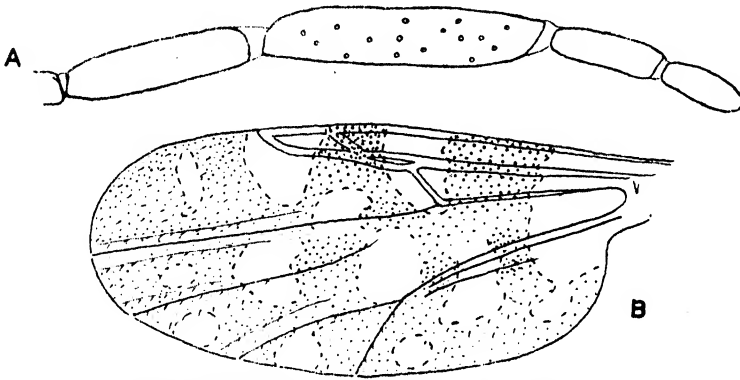


FIG. 3. *Culicoides palpalis* sp. nov., female. A.—Palp; B.—Wing.

subcylindrical, not inflated, without pit, but bearing sensory hairs scattered sparsely over greater part of length. Antennae missing.

Thorax very dark brown above, mottled with large and rather indistinct paler areas. Scutellum very dark or blackish, bearing the usual 3–4 bristles and one or two small hairs.

Wings (fig. 3B) with adornment similar to that of *C. verecundus*, but pale spot near middle of cell R 5 quite small and reaching neither to wing margin nor to fold above vein M 1. Macrotrichia distributed much as in *C. verecundus*. Halteres with almost white knobs.

Legs rather dark brown. Foreleg with knee dark and a narrow pale band on each side of it; middle leg with knee pale and nearly half of femur and tibia on each side of it also pale, yellowish; hind legs missing. Fourth tarsal segment on four anterior legs not cordiform.

Abdomen dark brown, but cerci paler. Spermathecae two, well chitinized, oval or obovate, rather unequal, about 65μ by 52μ and 48μ by 41μ respectively; with no part of the duct chitinized. Usual rudimentary third spermatheca and chitinized ring present.

San Cristobal, north of Aurora, 1,000 m., 9 III 1938, 1 ♀, taken at night on veranda by means of a trap-lamp.

Culicoides pusillus Lutz

El Vergel, 28 V 1935, 2 ♀, 'at light,' and 5 VI 1935, 1 ♀, 'at light near house.'

Culicoides pampoikilus sp. nov.

A small dark-brown species, resembling *C. poikilonotus* in many ways, but with a small pale spot in cell R 5 at tip of wing anterior to end of vein M 1, and differing also as indicated below.

FEMALE. Length of wing about 1.2 mm., greatest breadth 0.4–0.5 mm.

Head very dark brown. Eyes bare, so far as can be seen without macerating the specimen. Palpi missing. Antennae darkish brown, segments showing an abrupt change of shape between 10 and 11; segments 4–10 oval, subequal, about 10 by 5–6 units; 11–14 more elongate, subequal, about 17–19 by 5–6 units; 15 about 29 by 5 units.

Thorax dark brown, conspicuously adorned above with large yellowish patches much as in *C. poikilonotus*. Scutellum dark brown, bearing the usual four bristles and a few small hairs.

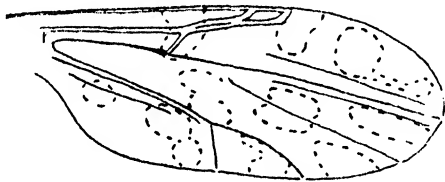


FIG. 4. *Culicoides pampoikilus* sp. nov. Wing of female.

Wings (fig. 4) with adornment somewhat similar to that of *C. poikilonotus*. The following differences, however, may be noted: pale spot near middle of cell R 5 rounded and not reaching anterior margin of wing (as in *C. poikilonotus*), but with a narrow extension from its posterior border towards wing-tip, where a small and rather indistinct pale area is formed just above tip of vein M 1; no pale spot overlying basal part of vein M 1, as there is in *C. loughnani* var. *jamaicensis*; distal pale spots in cells M 1 and M 2 large, reaching to margin of wing; and two pale spots in distal part of anal cell more or less connected to form a bilobed pale area. The part of the wing lying immediately posterior to the radial cells is dark, without either a pale spot or a pallid zone. Macrotrichia abundant, covering practically whole wing surface except radial areas, and numerous in cell Cu 1 and in anal cell. Halteres with pale knobs.

Legs rather dark brown, much damaged. T.R. about two. Fourth segment of tarsi of hind legs (others missing) small, but not cordiform.

Abdomen darker brown than thorax. Spermathecae two, well chitinized, pyriform, subequal, about 40 μ by 26 μ . Rudimentary oval third spermatheca and ring present.

El Vergel, 28 V 1935, 1 ♀, and 4 VI 1935, 1 ♀, taken at night at light.

Following the key given by Hoffman (1925) to the species of *Culicoides* of North and Central America and the West Indies, and that of Root and Hoffman (1937) to the North American species of the same genus (which includes Mexican species), this insect runs down to *C. stellifer* Coq. The figures of the wing of *C. stellifer* given by Malloch (1915) and Hoffman (1925) differ somewhat, but they agree in showing two pale spots in the distal part of cell M 1, instead of the single pale spot found in the species described above. Moreover, macrotrichia are less abundant in *C. stellifer*, since, according to Hoffman, there are 'but few macrotrichia other than above vein M 1' in the female.

It may be convenient to correct here my identification in 1937 of some specimens from Trinidad as *C. stellifer* Coq. They are, I believe, the same as those (also from Trinidad) described by Fox (1946) and named by him *C. stubalensis*, and the same as those from French Guiana described by Floch and Abonnenc (1942) and named by them *C. guyanensis*. They would seem to resemble closely *C. pictipennis* (Philippi), an insect found in Chile and described in 1865; but even if they should prove to be the same species the name *pictipennis* would not be available for them because it is preoccupied by Staeger's (1839) species.

***Culicoides loughnani* var. *jamaicensis* Edw.**

El Vergel, 28 V 1935 to 9 VI 1935, 1 ♂, 10 ♀, at light at night; Huixtla railway station in coastal plain, 4 VII 1935, 1 ♀, at light in hotel in evening; and San Geronimo, Indian village in Huixtla valley, 11 III 1938, 1 ♀, taken at night by means of a trap-lamp by the river, facing rocky mountain slope with virgin forest.

This species has been found at Ancon as well as in Jamaica. The following details about it, taken from the examination of these Mexican specimens (females), will be of service in separating it from some of the other somewhat similar species found in Dr. Dampf's collection. The single male is unfortunately damaged, lacking the end of the abdomen and the antennae.

Length of wing about 1.2 mm. Eyes bare, narrowly separated above. Palpi very dark brown; third segment much inflated, with large deep pit; lengths of last three segments in one specimen about 24, 7 and 9 units respectively. Antennae pale brown, showing a definite change of shape between segments 10 and 11; segments 4-10 subspherical to oval, ranging in one specimen from 7 by 8 to 10 by 7 units; 11-14 longer, subequal, in the same specimen about 16-17 by 6-8 units; 15 about 23 by 7 units, with rather blunt ending. Thorax not conspicuously adorned, and not spotted. Macrotrichia abundant on wings, covering most of their surface, extending to base between veins M and Cu, and numerous in anal cell. Legs with knees dark, a narrow pale band on each side of them. Fourth tarsal segments not cordiform. T.R. about two. Spermathecae two, well chitinized, subspherical to oval, subequal, in one specimen measuring 45μ by 38μ and 37μ by 34μ respectively. No part of the commencement of the duct is chitinized. There is a small chitinized ring on the duct, and a somewhat vermiform rudiment of a third spermatheca.

This species must be distinguished from *C. arboricola* Root and Hoffman, a somewhat similar North American species, which differs, however, in wing adornment by having the pale spot enveloping the cross-vein much larger, extending well over the vein M, the tip of vein Cu 2 pale-margined, and a large pale area at the base of the wing.

Culicoides alambiculorum sp. nov.

A small species resembling *C. loughnani* var. *jamaicensis* Edw. but differing as indicated below.

FEMALE. Length of wing about 1.0 mm., greatest breadth 0.4 mm.

Head very dark brown. Eyes narrowly separated above, bare, so far as can be seen without macerating the specimens. Palpi dark brown; third segment greatly inflated, with very large and deep pit; lengths of last three segments about 18, 7 and 7 units respectively. Antennae brown; segments 4–10 from subspherical to oval, measuring in one specimen from 7 by 7 to 9 by 6 units; 11 about 12 by 6 units; others missing.

Thorax dark brown, not conspicuously adorned and not spotted. Scutellum dark brown, bearing the usual 3–4 bristles and a few small hairs.

Wings (fig. 5) with adornment similar to that of *C. loughnani* var. *jamaicensis*, but pale spot, which in that species overlies basal part of M 1, reduced to a small but distinct pale area in cell R 5 which does not extend over the vein into cell M 1, and in distal part of anal

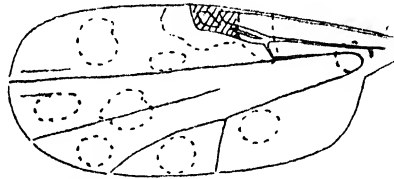


FIG. 5. *Culicoides alambiculorum* sp. nov. Wing of female.

cell only a single pale spot. Macrotrichia abundant, covering practically whole wing surface except radial areas and quite numerous in cell Cu 1 and in anal cell.

Legs darkish brown, with dark knees bordered on each side by paler bands. T.R. about two. Form of segments normal; fourth segments of tarsi subcylindrical.

Abdomen dark or darkish brown. Spermathecae two, well chitinized, retort-shaped, unequal, body portions in one specimen about 40μ by 37μ and 29μ by 24μ respectively.

El Vergel, 28 V 1935, 2 ♀, 4 VI 1935, 1 ♀ and 5 VI 1935, 1 ♀ (? teneral), taken at night near the house by means of a trap-lamp; and San Cristobal, 9 III 1938, 2 ♀.

Culicoides propinquus sp. nov.

A small species resembling in some respects *C. albomacula* Root and Hoffman, but with distal pale spot in cell R 5 remote from tip of wing, with the thorax not conspicuously adorned, and with wing markings as shown in fig. 6A.

MALE. Length of wing 0.9–1.0 mm., greatest breadth 0.3–0.4 mm.

Head dark brown. Eyes apparently bare. Palpi darkish brown; third segment inflated, with deep pit. Antennae rather pale brown but segments 13–15 more infuscated, short, plume scanty; lengths of last four segments about 6, 22, 17 and 22 units respectively.

Thorax dark brown above, not conspicuously adorned and not spotted. Scutellum dark brown, bearing the usual 3–4 bristles and one or two small hairs.

Wings with short radial cells, second and nearly half first enveloped in a dark area. Adornment as shown in fig. 6A. Pale spot covering cross-vein large, extending posteriorly

to about midway between veins M and Cu. Two pale spots in cell R 5, both large, the distal one scarcely reaching to either wing margin or fold above vein M 1. No pale zone or spot immediately posterior to radial cells; no pale spot straddling basal part of vein M 1, and no pale spot at tip above end of vein M 1. Vein M 2 straddled by a double pale spot, each half of which is connected by a pallid line with the pale area in the middle of the wing. Two pale spots in distal part of anal cell nearly confluent. Macrotrichia fairly abundant, distributed rather sparsely over wing distal to level of end of costa; a few also in anal cell. Halteres with almost white knobs.

Legs darkish brown. Knees dark on four anterior legs, with narrow pale band on each side, but on hind legs femora entirely darkish brown. T.R. about two. Fourth tarsal segments subcylindrical, not cordiform.

Abdomen darkish brown. Hypopygium with ninth tergite broad posteriorly, apparently not cleft, with long, finger-like, apico-lateral processes (fig. 6B); ninth sternite deeply excavated, membrane joining it to aedeagus not spiculate. Side-pieces and bases of claspers dark or darkish brown, tips of claspers paler, crooked at ends. Microtrichia covering about basal halves of claspers. Basal processes of side-pieces rather long, sub-

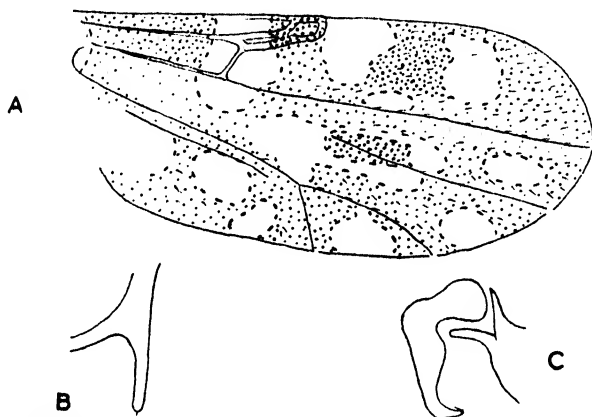


FIG. 6. *Culicoides propinquus* sp. nov., male. A.—Wing; B.—Apico-lateral process of ninth tergite; C.—Harpe.

equal, the inner ones not shaped like a boat-hook, as they are in *C. albomacula*. Harpes (fig. 6C) separate but contiguous at base, where they are much expanded and bent almost at a right angle; tips of harpes tapering, twisted, with pointed ends which may perhaps be subdivided. Aedeagus forming a wide basal arch with a gutter-like posterior extension.

San Cristobal, north of Aurora, 1,000 m., 9 III 1938, 1 ♂, taken at night on a veranda by means of a trap-lamp.

***Culicoides poikilonotus* sp. nov.**

A small species with the mesonotum conspicuously adorned with dark and yellowish patches, and with the wings somewhat similar to those of *C. loughnani* var. *jamaicensis* Edw., but differing from them as described below.

FEMALE. Length of wing about 1.1 mm., greatest breadth 0.4 mm.

Head very dark brown. Eyes bare, so far as can be seen without macerating the specimen. Palpi short; lengths of last three segments about 12, 5 and 6 units respectively; third inflated, with large and deep pit. Antennae missing.

Thorax brown, mesonotum conspicuously adorned with large yellowish patches arranged somewhat as shown in Lutz's (1913) figure of his *C. reticulatus*. Scutellum also parti-coloured, bearing the usual three bristles and one or two small hairs.

Wings with adornment as shown in fig. 7. It is of the type found in *C. loughnani* var. *jamaicensis* Edw., but the pale spot straddling basal part of vein M 1 in that species is here reduced to a rather indistinct pale area in cell R 5 only, and distal pale spot in cell R 5 situated closer to pale spot just beyond end of costa. Cross-vein not infuscated, the pale spot enveloping it extending posteriorly only just beyond vein M. Distal pale spots in cells M 1 and M 2 set back from margin of wing. Macrotrichia abundant, covering greater part of wing and so numerous in cell Cu 1 and in anal cell. Halteres with pale knobs.

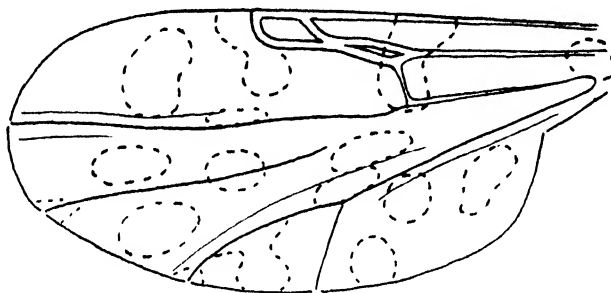


FIG. 7. *Culicoides poikilonotus* sp. nov. Wing of female.

Legs darkish brown, with blackish knees bordered on each side by narrow pale bands; tibiae of hind legs with broader subapical pale bands also. T.R. about two. Form of segments normal; fourth segments of tarsi subcylindrical.

Abdomen darker brown than thorax. Spermathecae two, moderately well chitinized, collapsed in the unique specimen but apparently of medium size, rather unequal, obovate, with commencement of duct chitinized for only a short distance, about 5μ .

El Vergel, 28 V 1935, 1 ♀, taken at night by means of a trap-lamp.

***Culicoides daedalus* sp. nov.**

A small species with the mesonotum conspicuously adorned, resembling the last species, *C. poikilonotus*, but differing as noted below.

MALE. Length of wing about 1.0 mm., greatest breadth 0.37 mm.

Head very dark brown. Eyes bare, so far as can be seen without macerating the specimen. Palpi short; lengths of last three segments about 12, 6 and 6 units respectively, the third inflated, with a large and deep pit. Antennae missing.

Thorax brown, with conspicuous adornment similar to that of *C. poikilonotus*. Scutellum also parti-coloured, bearing three bristles and a few small hairs.

Wings with adornment as shown in fig. 8A; very similar to that of *C. poikilonotus*, but without trace of pale spot at base of vein M 1, and with distal pale spot in cell R 5 separated more widely from pale spot at end of costa; resembling also that of *C. propinquus*, but with pale spot enveloping cross-vein smaller. Macrotrichia numerous in cell R 5, near tip in cells M 1 and M 2, and along veins Cu 1 and Cu 2, but none, or only an odd one or so, in cell Cu 1 and none in anal cell. Halteres with pale knobs.

Legs darkish brown, with dark knee-spots bordered on each side by narrow pale bands. Hind legs missing. Form of segments of other legs normal.

Abdomen paler brown than thorax. Hypopygium dark brown, resembling that of *C. simulans* Root and Hoffman, a North American species with an entirely different wing pattern, but with posterior extension of aedeagus shorter. Ninth segment with sternite

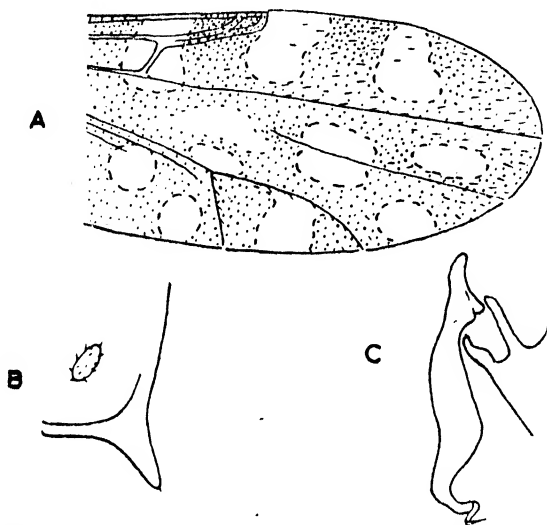


FIG. 8. *Culicoides daedalus* sp. nov., male. A.—Wing; B.—Apico-lateral process of ninth tergite C.—Harpe.

moderately excavated, the membrane joining it to aedeagus not spiculate; tergite (fig. 8B) cleft in middle line posteriorly, with large apico-lateral processes. Side-pieces longer and narrower than those of *C. simulans*, but with similar basal processes. Claspers bearing microtrichia on basal third or half only. Harpes (fig. 8C) similar to those of *C. simulans*, with twisted ends tapering to fine points. Aedeagus with well chitinized limbs forming a wide arch, and median posterior extension feebly chitinized, almost colourless, short, with rather blunt end.

El Vergel, 9 VI 1935, 1 ♂, taken at night by means of a trap-lamp.

***Culicoides propriipennis* sp. nov.**

A species of medium size, with the thorax rather conspicuously adorned, the wings characteristically marked, and the two spermathecae feebly chitinized.

FEMALE. Length of wing about 1.3 mm., greatest breadth about 0.5 mm.

Head dark brown. Eyes bare, so far as can be seen without macerating the specimen. Palpi and antennae missing.

Thorax rather dark brown above but conspicuously adorned with large, paler, yellowish areas.

Wings dark, adorned with rather small, sharply defined, pale areas arranged as shown in fig. 9. Radial cells large and widely open. Macrotrichia fairly numerous at tip but restricted to the part of the wing distal to level of end of costa.

Legs missing.

Abdomen darkish brown. Spermathecae two, feebly chitinized, collapsed in the unique specimen but probably obovate and about 48μ by 30μ ; the duct narrow and chitinized at its commencement for a considerable distance, about 18μ .

San Cristobal, north of Aurora, 1,000 m., 9 III 1938, 1 ♀, taken at night on a veranda by means of a trap-lamp.

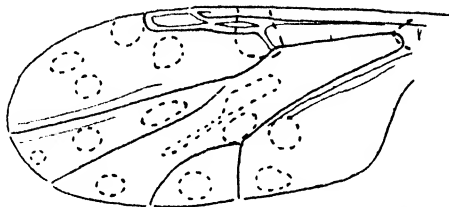


FIG. 9. *Culicoides propriipennis* sp. nov. Wing of female.

Although greatly damaged, this insect is briefly described because of its characteristic wing adornment and rather unusual spermathecae. From other species in which the second radial cell is completely enveloped in a dark area, and in which three distinct pale spots are present in cell M 1 (e.g., *C. paraensis* Lutz, *C. guyanensis* Floch and Abonnenc, *C. stubalensis* Fox), this species differs in having no pale spot near the tip of the wing in cell R 5.

Culicoides cacozelus sp. nov.

A small species closely resembling the species described above (p. 82) as *C. poikilonotus*, but with the pale spot near the middle of vein M 2 of the wing not straddling this vein, but reduced to a pale area in cell M 1 only.

FEMALE. Length of wing about 1.0 mm., greatest breadth 0.4 mm.

Head very dark brown. Eyes bare, so far as can be seen without macerating the specimen. Palpi pale brown, short; lengths of last three segments about 12, 6 and 6 units respectively, the third inflated, with large and deep pit. Antennae missing, except for segments 3-7 on one side, which show no characteristic features.

Thorax brown, conspicuously adorned, as in *C. poikilonotus*. Scutellum also particoloured, bearing three bristles and a few small hairs.

Wings (fig. 10) with adornment as in *C. poikilonotus* with one notable exception—the pale spot situated near middle of vein M 2 does not straddle that vein, but is reduced to an oval pale spot situated entirely in cell M 1. Macrotrichia abundant, covering most

of wing rather sparsely, and so numerous in cell Cu 1 and in anal cell. Halteres with pale knobs.

Legs darkish brown, with dark knees and other adornment as in *C. poikilonotus*. T.R. about two. Form of segments normal.

Abdomen a little darker than thorax. Spermathecae two, moderately well chitinized, partially collapsed in the unique specimen but apparently obovate, subequal, about 42μ by 27μ , tapering to duct, which is chitinized for only a short distance.

El Vergel, 5 VI 1935, 1 ♀, taken at night by means of a trap-lamp.

Apart from the single notable difference in the adornment of the wings, this species and the one described earlier (p. 82) as *C. poikilonotus* are so similar that it may be questioned if they are distinct species or two forms of a single species. Here I have regarded them as distinct, pending the examination of further specimens, especially males.

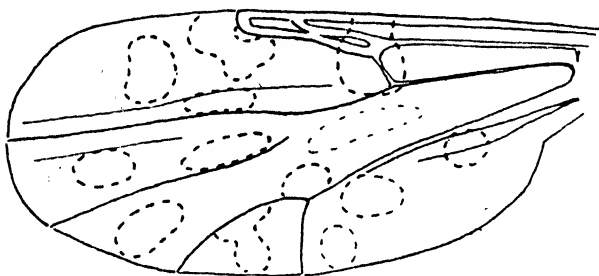


FIG. 10. *Culicoides cacoelus* sp. nov. Wing of female.

Culicoides eublepharus sp. nov.

This species is included here for the sake of completeness and in order that it may be placed in the key. It was erected for a single female, taken in British Guiana, found in a collection of Ceratopogonidae made by Mr. C. A. Hudson during the months of February and March, 1938, but was not included in my report on the rest of the collection (1940) because the specimen was lost.

The insect closely resembled *C. pulchripennis* Macfie, but the eyes were rather densely hairy instead of being bare, as they are in that species. The third palpal segment was inflated and furnished with a large shallow pit in its distal half. The antennae were missing. The adornment of the hind legs and the form of the single spermatheca were as in *C. pulchripennis*. The wings, too, were similar as regards venation and the distribution of macrotrichia, and showed only slight differences in the adornment—e.g., the pale spot at the end of the costa was divided into two, the pale area in the middle of the wing reached further posteriorly so as to lie over the bifurcation of the vein Cu, and the pale spot situated between the branches of Cu was somewhat smaller.

Culicoides debilipalpis Lutz

El Vergel, 28 V 1935, 2 ♀, and 11 VI 1935, 1 ♀; Finca Palenque, 1,000 m., in virgin forest, 10 III 1935, 1 ♀ (L. Grajales); and Finca Esperanza, 27 VI 1938, 1 ♀ (R. Nettel).

These insects are probably the same as those from Trinidad which (in 1937) I referred to this Brazilian species. The eyes are apparently bare, or nearly bare. The antennal segments 4-15 form an almost continuous series, without any distinct change of shape between 10 and 11. The wings bear numerous macrotrichia arranged more or less as shown in Lutz's (1913) figure, with a double row extending nearly to the base between M and Cu; they are therefore more abundant than they are in *C. horticola* Lutz, *C. glabrior* M. or *C. germanus* M. The third palpal segment is furnished with a well-developed pit, which is rather larger and deeper than that shown in da Costa Lima's (1937) figure of the palp of *C. debilipalpis*, but smaller than that of *C. glabrior* according to my (unpublished) sketches.

In a key to the females of the Caribbean species of *Culicoides* published by Fox (1946) *C. debilipalpis* has unfortunately been misplaced among species in which the second radial cell is 'mainly included in a light spot.' But *C. borinqueni*, a Puerto Rican insect briefly described by Fox and Hoffman (1944), is listed; it appears to resemble *C. debilipalpis* closely and may be the same species.

The species may be composite. It is not yet possible to make a comparison of the characters of the hypopygium of males from the different localities, because hitherto in most of them only females have been collected.

THE CULTIVATION OF EXOERYTHROCYTIC FORMS OF *PLASMODIUM GALLINACEUM*

I.—A PRELIMINARY NOTE

BY

G. GRAMICCIA*

AND

ROBERT H. BLACK†

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)

(Received for publication February 10th, 1948)

The following note describes some of our observations on the development of exoerythrocytic forms of *Plasmodium gallinaceum* grown in tissue culture. The method used was based to some extent on that described by Hawking (1945). The medium contained fowl serum, Tyrode's solution, chick-embryo extract and penicillin. Schizonts were seen within the macrophage cells migrating from the primary implant in a tissue culture of chick's spleen.

The chick used had been infected by intravenous inoculation of blood containing trophozoites of *P. gallinaceum* when it was six days old. Quinine, 2.5 mgm., was given orally each day from the date of inoculation until the end of the prepatent period, when the dose was doubled. The parasitaemia persisted, and there were still scanty parasites in the blood on the day on which the culture was begun. Exoerythrocytic schizonts were found in the spleen and brain at the time when the culture was set up.

Typical exoerythrocytic schizonts within a macrophage cell are shown in Plate IV, fig. 1, in a specimen removed after eight days' incubation at 37° C. To examine a culture, a fragment of cover-slip was removed from the Carrel flask, and the central portion of tissue was removed and made into a smear. The material remaining on the cover-slip represented, in the main, cells which had grown during the period of cultivation. The tissues were fixed while wet in Zenker's fluid. In fig. 1 the parasites are in a macrophage within the area of migration of cells, well away from the original implant.

DISCUSSION

Cells in which exoerythrocytic forms of *P. gallinaceum* develop in tissue culture show morphological differences from those seen to be parasitized in the infected chick. These cells, however, retain their power of migration and their phagocytic properties, the latter being illustrated in Plate IV, fig. 2, where numerous red cells can be seen within a macrophage. The cells which were seen to be infected in the cultures resembled some of those depicted by Parker (1938) for incubated chick leucocytes, in that vacuolation of the cytoplasm was a notable feature. We have called the cells containing the exoerythrocytic schizonts 'macrophages,' but their appearance in tissue culture does not allow an accurate diagnosis of cell type to be made. Even in tissues the type of cell invaded is difficult to

* World Health Organization Fellow.

† Working under a grant from the Medical Research Council.



FIG. 1. Exoerythrocytic schizonts within a macrophage cell.

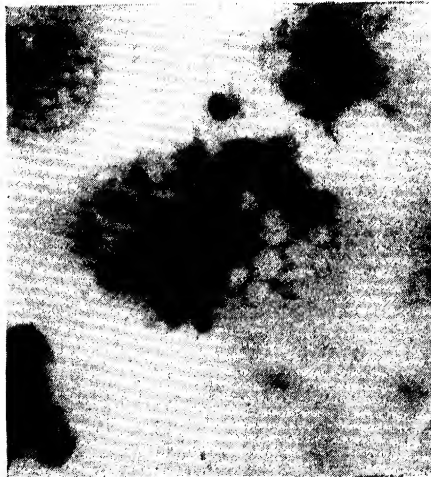


FIG. 2. Macrophage cell containing numerous ingested erythrocytes.

determine. It does appear, however, that the exoerythrocytic forms of the various species of the genus *Plasmodium* have a definite host-cell specificity: thus, the exoerythrocytic forms of *P. elongatum* invade cells of the haemopoietic system, while its trophozoites are actively phagocyted by the macrophages; *P. gallinaceum* blood forms are not found phagocyted by the macrophages, but its exoerythrocytic forms invade cells of the reticulo-endothelial system, even when the latter show morphological differences and a somewhat different behaviour in the artificial medium in which they live and grow, as in a tissue culture; Shortt *et al.* (1948*a*) consider that the cells invaded in the liver by the early exoerythrocytic stage of *P. cynomolgi* are possibly the polygonal cells. These workers (1948*b*) have also demonstrated the similar stage of *P. vivax* in the human liver.

ACKNOWLEDGEMENTS.—Dr. Gramiccia expresses his grateful acknowledgements to Dr. F. Hawking for instruction given in the technique of tissue culture of plasmodia.

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THE RESORPTION OF HAEMOGLOBIN BY THE RENAL TUBULES IN HAEMOGLOBINURIA

BY

ROBERT H. BLACK*

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)

(Received for publication February 10th, 1948)

In a study of the kidneys of dogs which had died with haemoglobinuria in *Babesia canis* infections, a prominent feature found was the presence of eosinophilic granules in the cells of some of the convoluted tubules. The same feature was seen in the kidney of a monkey which had died from *Plasmodium knowlesi* infection with haemoglobinuria. The granules gave the haemoglobin staining reaction with cyanol. Other forms of haemoglobinuria were therefore investigated to see if these phenomena were a constant finding of the condition.

Similar granules were found in tubular cells of the kidneys of a number of animals and man in conditions of haemoglobinuria due to a variety of causes, and were identified as haemoglobin. Further, it was observed that these granules were released into the tubular lumina when the cells containing them degenerated, and thus they came to form part of the tubular debris. These changes were also seen in a mouse after the intravenous injection of lysed blood.

MATERIAL

Kidneys from the following animals were examined:

1. Dog, *B. canis* infection.
2. Monkey, *P. knowlesi* infection.
3. Rat, phenylhydrazine poisoning.
4. Rat, *Bartonella muris* infection.
5. Man, blackwater fever.
6. Man, incompatible transfusion.
7. Mouse, intravenous injection of lysed blood.

B. canis. Puppies were infected with the strain maintained at the Liverpool School of Tropical Medicine. After passage through puppies the virulence became exalted, so

PLATE V

ALL THE SECTIONS DEPICTED WERE STAINED WITH HAEMATOXYLIN AND EOSIN

FIG. 1. *B. canis*. Granules within the epithelial cells of the convoluted tubules. The cells are degenerate, but none of the granules have been shed into the lumen.

FIG. 2. *B. canis*. Further swelling and degeneration of the tubular cells, with shedding of cellular debris and granules into the tubular lumen.

FIG. 3. *B. canis*. A medullary tubule, with normal epithelial cells, containing granules within its lumen.

FIG. 4. *B. canis*. Granules within tubular cells of the medulla.

FIG. 5. *B. canis*. General view of kidney, to show the patchy distribution of the convoluted tubules which contain intracellular granules.

FIG. 6. *P. knowlesi*. Granules are seen within the degenerate tubular epithelial cells; some have also been shed into the lumina of the tubules.

* Working under a grant from the Medical Research Council.

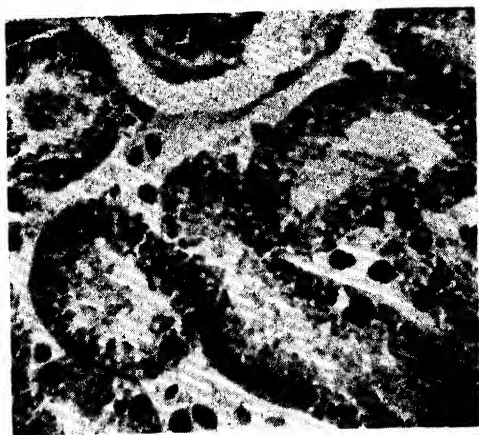


FIG. 1

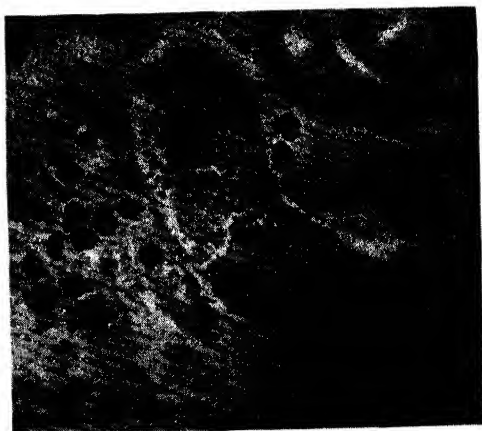


FIG. 2

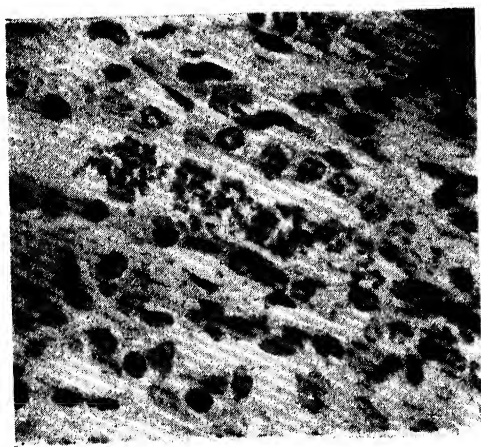


FIG. 3

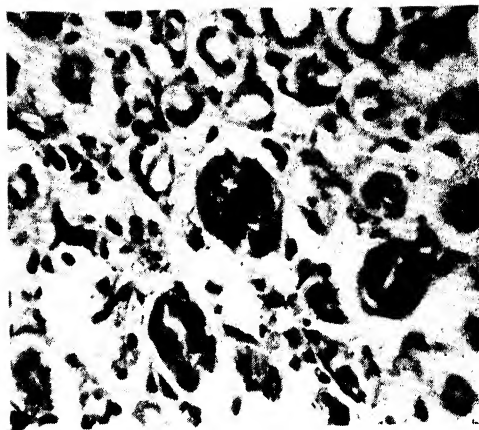


FIG. 4

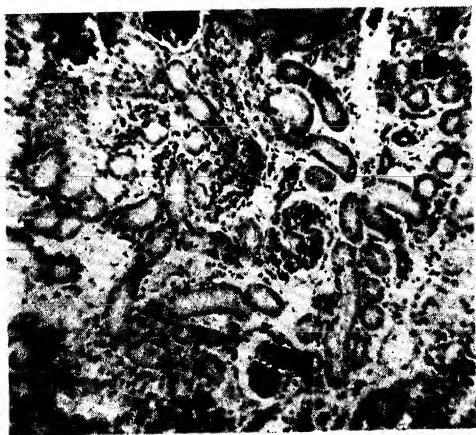


FIG. 5

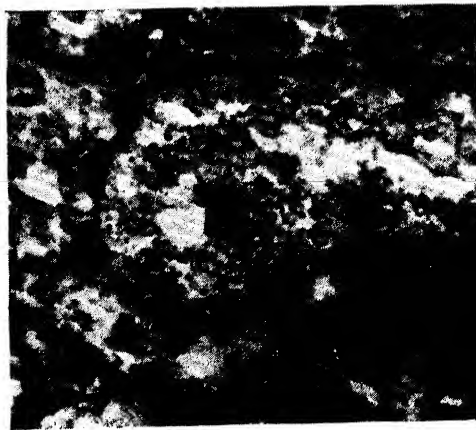


FIG. 6

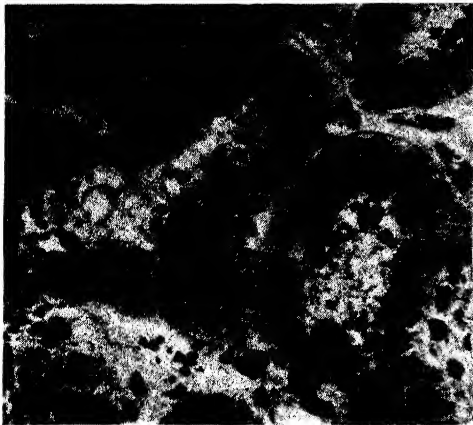


FIG. 7

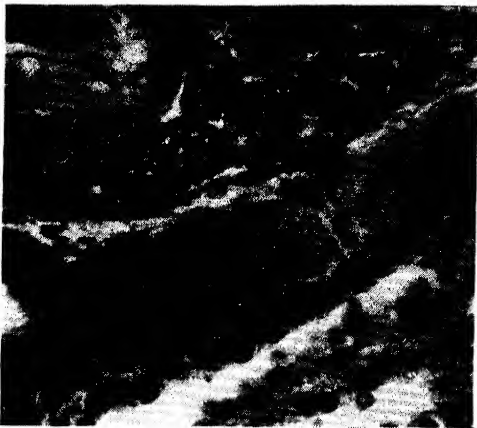


FIG. 8

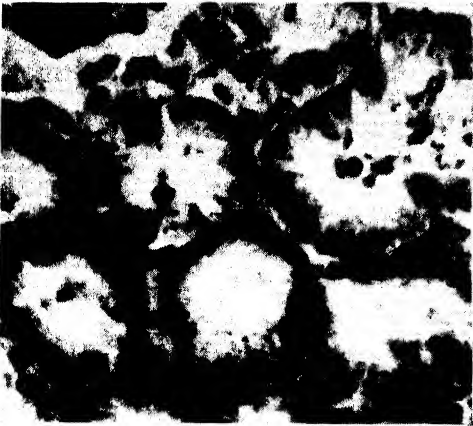


FIG. 9

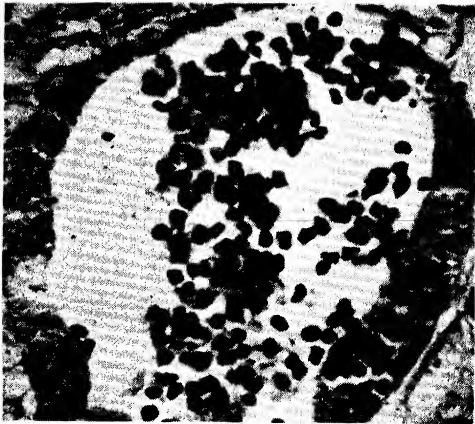


FIG. 10

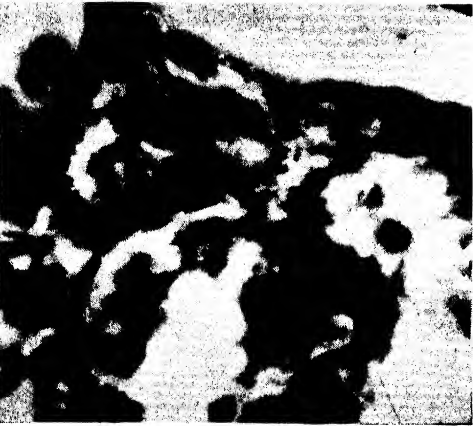


FIG. 11

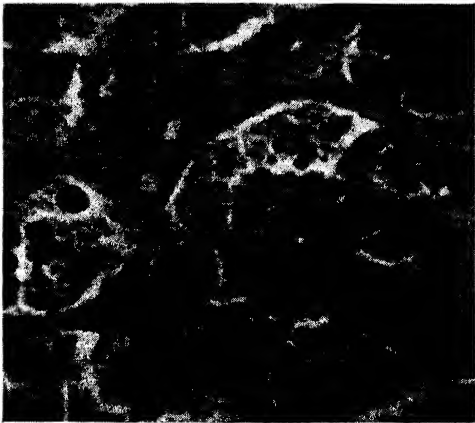


FIG. 12

that death of the animals occurred with haemoglobinuria within a few days of intraperitoneal injection of infected blood.

P. knowlesi. A *rhesus* monkey was examined which had died from an acute exacerbation of a chronic and partially treated infection. Its urine contained haemoglobin pigment *post mortem*.

Phenylhydrazine. A rat was injected subcutaneously with a solution of phenylhydrazine hydrochloride dissolved in 0.85 per cent. saline. A series of five injections with doses ranging from 7 mgm. to 28 mgm. was given over a period of 11 days. The rat died on the 11th day.

B. muris. The spleen was removed from a stock white rat. Haemoglobinuria developed and the rat died on the fifth day after operation, with its red blood-cells showing numerous Bartonellae.

Blackwater fever. The section examined was from the collection of the Liverpool School of Tropical Medicine.

Transfusion kidney. The sections examined were cut from a block kindly lent by Professor A. C. Lendrum, of the University of St. Andrews. No history of the patient is to hand, apart from the fact that death was due to an incompatible transfusion.

Intravenous injection of lysed blood. Blood was taken from a mouse by cardiac puncture, and was defibrinated and lysed by alternately freezing with solid CO₂ and thawing in warm water. When completely lysed, the blood was centrifuged to remove the stroma, and 0.3 ml. was injected into the tail vein of a mouse. The mouse was killed 48 hours later.

RESULTS

The tubular changes are well shown in Plates V and VI. Eosinophilic granules appear mainly in the convoluted tubular cells, but may even be seen in the tubules of the medulla (fig. 4). These granules are very striking in appearance, and stain a bright red colour with eosin. The cells may be seen to be almost packed with them. The granules vary in size, measuring up to about 3μ in diameter and being smallest in the rat. The next change seen is the swelling and disintegration of the tubular cells. The granules are then released into the tubular lumen and form part of the debris found in the tubules, where conglomerates of these rounded granules may be seen (see figs. 3 and 8). Usually all the tubules are not affected. Fig. 5 shows the general appearance of a section in *B. canis* infection in which only scattered convoluted tubules contain granules.

PLATE VI

ALL THE SECTIONS DEPICTED WERE STAINED WITH HAEMATOXYLIN AND EOSIN

FIG. 7. *Transfusion Kidney, Man.* Granules, which vary markedly in size, are seen within the epithelial cells of convoluted tubules. Some of these cells have degenerated and discharged the granules into the tubular lumina. (There are some particles of dust in the section.)

FIG. 8. *Transfusion Kidney, Man.* A tubule of Henle's loop containing granules. An adjacent convoluted tubule has its cytoplasm packed with granules.

FIG. 9. *Rat, Phenylhydrazine Poisoning.* Granules can be seen both within the cytoplasm of the tubular cells and within the lumina of the tubules.

FIG. 10. *Blackwater Fever.* Granules are seen within the epithelial cells of a distended tubule. The release of granules from the degenerating epithelium is well shown.

FIG. 11. *Bartonella muris, Rat.* There are granules within the tubular cells, and many have been released into the lumina of the tubules with the degeneration of the epithelial cells.

FIG. 12. *Intravenous Injection of Lysed Blood, Mouse.* Granules within the cells of the tubular epithelium. The tubule also contains a non-granular cast.

In the blackwater fever kidney examined some searching of the section was required before the granules were seen. On the other hand, fig. 4 shows that the granules may be very numerous and may be found even within the tubular cells of the medulla.

The legends to the figures indicate the changes seen in the various conditions studied.

Determination of the composition of the granules. The following staining reactions were observed: haematoxylin and eosin—eosinophilic; Prussian blue—granules did not stain blue; carbol-fuchsin and light green—violet; Heidenhain's iron alum haematoxylin—dark purple, as with red cells; cyanol (Dunn, 1946)—blue; Lison (1931) leuco-fuchsin—mauve (after heating the section to 100° C. for 20 minutes); Okajima (1916) phosphomolybdic acid and alizarin sodium sulphonate (alizarin red S)—orange; Kindred's (1932) modification of Okajima's stain with iron alum as mordant—violet.

All these staining reactions are in agreement with the conclusion that the granules are haemoglobin. The Lison (1931) and the Dunn (1946) stains depend upon the oxidation of compounds from their leuco- (reduced) state to the oxidized (coloured) state by haemoglobin. Lison (1931) stated that the leuco-derivative of acid fuchsin was oxidized to the coloured compound by both haemoglobin and the peroxidase granules of leucocytes, but that this property was retained only by haemoglobin after heating to 100° C. for 20 minutes, as was observed with these intracellular granules.

DISCUSSION

The results of these experiments have shown that, in these conditions associated with haemoglobinuria, granules of haemoglobin are found within the epithelial cells of the convoluted tubules. With degeneration of these tubular cells the granules are released into the lumina, where they form a prominent part of the tubular debris.

Similar intracellular granules have previously been described in association with haemoglobinuria. Stieda (1893) described granules in the epithelium of convoluted tubules in blackwater fever, and stated that they gave an iron reaction. Marchiafava and Bignami (1900) also reported finding granules in the same disease and called them haemoglobin granules. They noted their similarity to those within the lumen of the tubules. Dudgeon (1920) noted granules in the tubular epithelium in blackwater fever, of which some gave an iron reaction and some did not. Stephens (1937) also described granules in this disease and referred to them as being haemoglobin, although some gave an iron reaction. In *Piroplasma (Babesia) canis* infections Yorke (1911) found granules in the tubular epithelium. They did not give an iron reaction and he presumed them to be haemoglobin, which was being secreted from the blood by the tubular cells and then excreted into the tubular lumen. He found similar granules in the kidney of the rabbit after intravenous injections of haemoglobin. Bordley (1931) described the presence of a few granules in tubular cells of the cortex after incompatible transfusion. This 'brownish pigmented material' was observed to increase in amount as the tubules were followed towards the pelvis of the kidney. Ayer and Gault (1942) found intracellular granular material in the tubules after incompatible transfusion and in cases of jaundice in infants. They did not identify the material. Harrison *et al.* (1947) described granules in dogs exposed to arsine and also after injection of haemoglobin. They concluded that the material was presumably haemoglobin or a derivative.

Several descriptions of the renal changes seen in haemolytic conditions associated with haemoglobinuria do not mention intracellular granules. Werner (1907), in the

cases of blackwater fever which he described, found no haemoglobin granules in the epithelial cells of the convoluted tubules. Barratt and Yorke (1909) found no intracellular granules in the kidneys of rabbits after intravenous injection of haemoglobin. Baker and Dodds (1925), describing two cases of incompatible transfusion, made no mention of these intracellular granules, though they were impressed by the eosinophilic granular material within the tubules. Terplan and Javert (1936), in a case of fatal haemoglobinuria due to an overdose with quinine, described haemoglobin globules in the collecting tubules but did not mention granules within tubular cells.

Yorke (1911) presumed that the granules found in the tubular cells in *B. canis* infections were formed from haemoglobin being actively excreted by these cells, but it appears from modern work (Monke and Yuile, 1940) that haemoglobin is excreted through the glomerulus. Lambert (1936) found granules, which he identified as haemoglobin, in the renal tubular cells of the salamander following subcutaneous injection of haemoglobin. Lison (1938) demonstrated the resorption of haemoglobin by the cells of the tubular epithelium of frogs after it had been injected through Bowman's membrane. In the tubular cells granules were formed which reacted with a haemoglobin stain. Hayman and Richards (1927) showed that the colloidal substance trypan blue was also absorbed through the luminal border of the tubular cells after it had been injected through Bowman's membrane. Monke and Yuile (1940) studied the excretion of haemoglobin in dogs and compared its rate of elimination with that of creatinine. They concluded that haemoglobin was absorbed from the glomerular filtrate by the proximal convoluted tubules at a maximum rate of 2 mgm. per minute, and that haemoglobinuria occurred when more than this amount was passed through the glomerular membrane.

Thus it appears to be established that haemoglobin can be absorbed by the tubular cells from the glomerular filtrate, and that in these cells the haemoglobin forms granules—a process which has been called athrocytosis. Ayer and Gauld (1942), however, regarded the appearance of 'brick-red material' within the epithelial cells of the renal tubules as a morphological manifestation of cell injury. This does not seem to be a tenable explanation in view of the evidence cited above. The haemoglobin granules within the tubular cells resemble the secretion granules of, for example, the pancreas. Whether the essential composition of the haemoglobin is altered or not remains a matter for speculation. Certainly the granules retain their peroxidase property and are able to convert the leuco-compound of cyanol into the coloured form. In the sections studied here, they were not dissolved in the glomerular filtrate when released into the tubular lumina. The degeneration of these cells containing the granules appears to be merely part of the more general cortical tubular necrosis which is a feature of these conditions of intravascular haemolysis with renal involvement and is well exemplified in blackwater fever.

The picture which is derived is that intravascular haemolysis results in the appearance of haemoglobin in the glomerular filtrate, from which it is absorbed by the convoluted tubules to form intracellular granular deposits. If there is more haemoglobin in the glomerular filtrate than the tubules can absorb the haemoglobin passes on down the tubules. Tubular degeneration is accompanied by the release of these granules into the lumina of the tubules, where they form aggregates amongst the tubular debris. The intravenous injection of lysed blood in the mouse resulted in degeneration of tubular cells which either did or did not contain haemoglobin. Thus the presence of haemoglobin granules within the cells is apparently not the essential cause of their degeneration.

The fate of the haemoglobin which is not resorbed by the tubules is not directly a concern of these experiments, and it is not possible to say to what degree the granules of haemoglobin from the tubular cells contribute to the granular debris seen within the tubules. The marked similarity in size and shape of the granules in the tubules suggests that much of this material has its origin within the tubular cells. When released from the degenerating tubular cells these granules of haemoglobin are obviously in a different physical state from that of the molecules of haemoglobin passing through the glomerular membrane. It is probable that the haemoglobin is modified by the tubular cells.

SUMMARY

1. In a variety of haemolytic conditions in animals and man eosinophilic granules are described and illustrated within the epithelial cells of the tubules of the kidney.
2. Similar granules are described in the mouse after intravenous injection of lysed blood.
3. These granules are identified as consisting of haemoglobin.
4. It is considered that this haemoglobin is derived from the glomerular filtrate.
5. Following degeneration of the tubular cells the granules are released into the lumina and form a prominent part of the tubular debris.

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THE PATHOGENESIS OF THE LIVER LESION DUE TO THE ADMINISTRATION OF CARBON TETRACHLORIDE

BY

W. H. HORNER ANDREWS

AND

B. G. MAEGRAITH

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)

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In the course of experiments designed to elucidate the pathogenesis of centrilobular changes in malaria (Maegraith *et al.*, 1947; Maegraith and Andrews, 1948) the effect on the liver of administering carbon tetrachloride, chloroform and phenylhydrazine was investigated. As has been repeatedly shown, both carbon tetrachloride and chloroform, if given in sufficient dosage, lead to centrilobular necrosis and degeneration (Lacquet, 1932; Takahashi, 1929; Phelps and Hu, 1924; Meyer and Pessoa, 1923). In this paper we provide evidence to show that the lesion is due, at least in part, to anoxia of the central region of the liver lobules, secondary to obstruction of the blood-flow through the sinusoids. This obstruction arises from swelling of the parenchymal cells, which leads to a diminished vascular bed within the liver (Himsworth, 1947*a*; Andrews and Maegraith, 1948).

The parenchymal cells at the centre of the hepatic lobule are especially vulnerable to both a slower flow of blood through the sinusoids and a reduction of the blood-oxygen tension, since the peripheral cells receive their requirements first. Any factor, therefore, which slows or reduces the blood-flow through the liver, which increases the metabolism of the hepatic cells, or which lowers the oxygen content of the blood passing to the liver will tend to produce centrilobular changes (Himsworth, 1947*a*; Rich, 1930; Chiari, 1899; Thompson and Turnbull, 1912; Resnik and Keefer, 1925-26; Rosin, 1928; Bainbridge and Leathes, 1906; Whipple and Hooper, 1916; Rous and Larimore, 1920; Behrend *et al.*, 1922; Bolton and Barnard, 1931; Simonds and Jergesen, 1935; Weatherford, 1935; MacCallum, 1928).

Maegraith and his colleagues believe that, in states of vasomotor dysfunction, a reflex constriction of the smaller radicles of the hepatic vein may take place, shock, malaria and crush injury being examples of the conditions in which this reflex can arise. It appears that this reflex may, on occasions, be present in carbon tetrachloride poisoning and play a part in producing the hepatic lesion.

Before this work was completed we learnt that Professor Himsworth had already demonstrated narrowing of the sinusoids in carbon tetrachloride poisoning, and we are indebted to him for many helpful suggestions.

METHODS

Animals

Albino rats were used for the majority of the experiments, and unless otherwise specified the descriptions of the lesions refer to this animal. Both carbon tetrachloride and chloroform were also administered to dogs, cats, rabbits, guinea-pigs and mice.

Dosage

A standard dose of 0.1 c.cm. per 100 gm. body-weight of carbon tetrachloride or chloroform was injected subcutaneously. When the dose was very small, as for mice, medicinal paraffin was used as a diluent, so that accurate dosage was made easier.

Injection of Sinusoids

Many methods of outlining the sinusoids were tried, including injection of dyes into the intact animal and perfusion of the excised liver with dyes, Indian ink or warm carmine gelatine. The method which gave the most reliable results was that of introducing Indian ink into the splanchnic system of the living animal.

After anaesthetizing the animal with ether a mid-line incision was made. In both rats and guinea-pigs the injection was made into one of the larger veins draining the caecal area. About 1 c.cm. per 150 gm. body-weight was introduced over a period of about a minute. The ink was filtered and rendered isotonic with sodium chloride, and was used at body-temperature. 'Mandarin' ink was found to be the most suitable, for the carbon particles do not readily conglomerate on injection (Himsworth, 1947b).

Before the injection was completed an incision was made in the diaphragm, and, on completion, clamps were placed simultaneously on the inferior vena cava, the aorta and the porta hepatis. Ligatures were then placed on the portal and hepatic veins and the liver was excised and placed in 10 per cent. formal saline for some hours (Bainbridge and Trevan, 1917).

Preparation of Tissues

Unless Indian ink was used, the smaller animals were killed by a blow on the occiput. Dogs and cats were killed either by hydrocyanic acid or under anaesthesia; the latter method did not appear to affect the state of the sinusoids and was preferable. The liver vessels were tied before the organ was excised and fixed as above. After varying length of time—depending on the size of the organ—the liver was sliced and more adequately fixed. Tissues for general histological examination were dehydrated and imbedded in the usual way.

When it was desired to demonstrate the sinusoids the liver slices were treated and cleared by the method of Reagan (1926). The clearing process may be hastened by putting the slices into benzyl benzoate at 50° C. for several hours. Sectioning was then done by hand.

Haemoglobin Estimations

Sahli's method was used throughout.

The Importance of Correct Methods

Unless the greatest care is taken, the picture seen under the microscope may not represent the state of the liver during life. Deep anaesthesia may result in splanchnic engorgement; failure to tie off the hepatic vessels may lead to draining of the sinusoids; the injection pressure must not be great enough to raise the portal venous pressure. To circumvent the last difficulty, and also to avoid handling the intestines, Himsworth (1947a) introduced the Indian ink into the spleen, but we found that this was not possible in guinea-pigs owing to the small size of the organ.

The sinusoidal pattern seen in thick cleared sections varies considerably from area to area. The necessity for taking a large number of sections from different sites need, therefore, not be emphasized.

RESULTS

The Hepatic Lesion

A description of the liver lesions will be given only in so far as it throws light on the pathogenesis. A fuller description is given by Cameron and Karunaratne (1936), who also review the literature to that date.

Macroscopically the colour of the liver varied from nearly normal to pale yellow. The latter colour was most evident in dogs. The lobular pattern was well marked, at times appearing as a dark-red mosaic on a pale background.

Microscopical changes became apparent after about six hours, when there was some swelling of the parenchymal cells throughout the lobule. The liver lesion was at its maximum 24-72 hours after the injection. At that stage the cells placed centrally in the lobule were degenerate and necrotic, staining well with eosin. The regular form of the liver cords was lost, although the reticulin framework remained intact. Occasionally haemorrhages were seen in this area. At a varying distance from the central vein, usually in the mid-zone, the parenchymal cells showed hydropic necrosis. The hydropic swelling was sufficient to distort the pattern of the liver cords, so that the bloated cells appeared to be flattened against each other, obliterating the sinusoids. More peripherally the sinusoids were greatly diminished by swelling of the parenchymal cells. In rats these cells were seldom hydropic, and apart from their enlargement appeared fairly normal. This was not the case with all animals. In rabbits, on a similar dosage, the picture was somewhat the same, but the portally placed cells were more hydropic, staining poorly. The lesion in the dog and the cat is essentially similar but much more severe than in the rabbit. The difference in severity may possibly be related to the diet, although the dogs received a liberal protein allowance.

The lesion in rats did not always show hydropic changes. Some degree of swelling was always present peripherally, but centrally the predominant lesion in a few cases was that of atrophy and degeneration.

The course of the lesion after the third day was towards recovery. Mitoses were present in many peripheral cells, and regeneration of the liver cords was inwards towards the central vein. Before the central debris was replaced, congestion and even haemorrhages appeared in the necrotic area. There was no indication, however, of stasis or thrombosis. After injection of trypan blue at this stage, thick sections cleared in cedarwood oil showed numbers of large mononuclear cells lying in advance of the regenerating parenchymal cells. Round-cell infiltration of the portal tracts was a common feature.

The appearance of the liver became almost normal, macro- and microscopically, after a week, and a few days afterwards was indistinguishable from that of a control animal.

The liver was markedly congested in two of the 50 rats used in this experiment. This congestion was present largely in the central veins and to a lesser degree in the sinusoids. In these cases the swollen parenchymal cells appeared to be pushed aside by pockets of blood. The erythrocytes were neither agglutinated nor sludged into homogenous masses.

In rats the hepatic lesion due to carbon tetrachloride or chloroform poisoning is not specific. We have observed similar acute lesions in poisoning by phenylhydrazine and butter yellow.

In the guinea-pig the lesion due to carbon tetrachloride poisoning is histologically different. Many of the livers examined showed early congestion, though this was often of slight degree only. Some swelling of the cells was apparent, but, except for isolated areas, the sinusoids were usually not obliterated. Centrilobular degenerative changes were seen, and in severe cases all but the periphery of the lobule showed necrosis. The necrosis was centrilobular, as in the other animals investigated. Hydropic necrosis was not, however, commonly seen unless the dose was raised, and when present it was not confined to a narrow zone, as frequently occurred in other species, as, for example, in the rat (Plate VII, fig. 1). In less severe cases most of the parenchymal cells of the lobule showed fatty changes towards the centre of the lobule. The subsequent resolution of the damage was similar to that in rats.

Blood-Haemoglobin Changes

Administration of phenylhydrazine may lead to a lesion similar to that due to carbon tetrachloride (Andrews and Maegraith, 1948). Anaemia is prominent in this case, and blood-haemoglobin concentrations were therefore measured in 10 rats to which carbon tetrachloride had been given. There was a small but definite fall in the haemoglobin concentration, lasting for 3-4 days, in eight of the animals. The maximum fall noted was 15 per cent., which is insufficient by itself to cause liver damage.

Narrowing of the Liver Sinusoids

In order to confirm the narrowing of the sinusoids observed in histological sections, Indian ink was used to outline the vessels (see Plates VII and VIII, figs. 3-6).

In the normal liver the sinusoids appear to be nearly equal in width to the liver cords, as is illustrated in fig. 3. It is not always possible to achieve such a complete injection, but in microscopical fields where the ink was not present in large amounts the sinusoids were outlined by the erythrocytes.

The sinusoids became considerably narrower after dosage with carbon tetrachloride. The narrowing was due to swelling of the parenchymal cells, which encroached on the space occupied by the vessels. In the earlier stages this swelling was most pronounced in the mid-zone (fig. 4). After 24 hours the calibre of the sinusoids was markedly reduced, except in the extreme peripheral areas (figs. 5, 6); large doses led to almost complete obliteration of these vessels.

Not all the sinusoids present showed gross alteration in size. Even in livers greatly affected by carbon tetrachloride a few sinusoids of almost normal calibre were seen. In rats these more normal channels occurred in leashes, but in guinea-pigs it was more usual to find a solitary 'normal' sinusoid surrounded by large numbers which were diminished in calibre. The existence of these channels would facilitate the drainage of the portal blood.

DISCUSSION

The centrilobular distribution of the hepatic lesion due to many poisons, including carbon tetrachloride, suggests that the agents do not act primarily by attacking the paren-

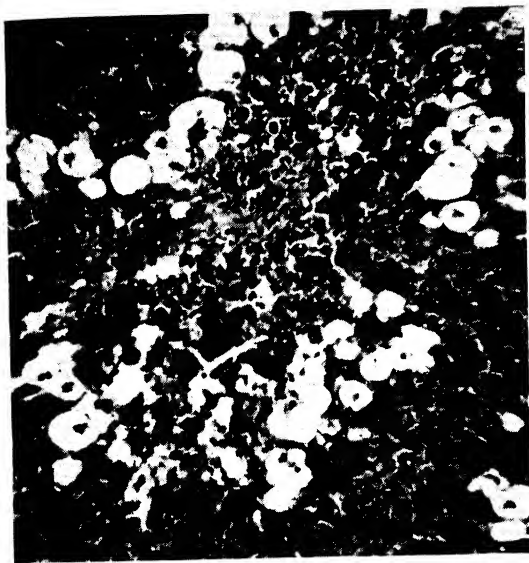


FIG. 1. Rat, 0.1 c.cm. CCl_4 per 100 gm. administered 30 hours previously. The peripheral cells are swollen and the sinusoids narrow. In the mid-zone there is an almost complete ring of hydropic necrosis. Centrally there are necrotic eosinophilic cells.

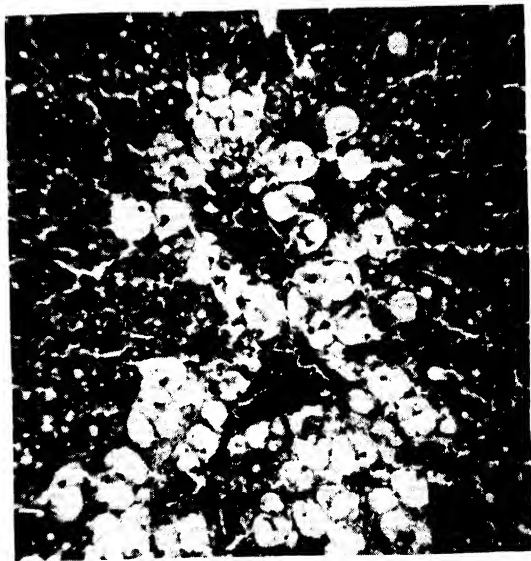


FIG. 2. Rat, 0.1 c.cm. CCl_4 per 100 gm. administered 24 hours previously. The two central veins in the field are dilated and congested. The hydropic necrosis is more central than in fig. 1.

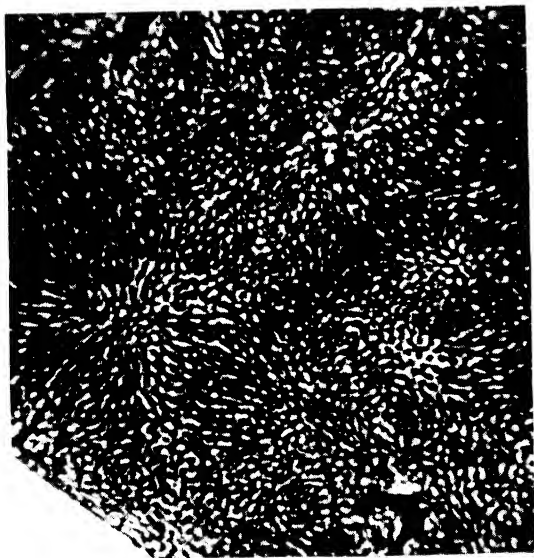


FIG. 3. Rat, normal. Indian ink introduced into the splanchnic system to outline the sinusoids.



FIG. 4. Rat, 0.2 c.cm. CCl_4 per 100 gm. administered 8 hours previously. The diminution in the calibre of the sinusoids (outlined in Indian ink) is predominantly mid-zonal.



FIG. 5. Rat, 0.1 c.cm. CCl_4 per 100 gm. administered 24 hours previously. There is a diminution in the calibre of the sinusoids (outlined in Indian ink), except peripherally.



FIG. 6. Rat, as in fig. 5, but double the dose was given and the magnification is greater. The sinusoids have been almost obliterated, except near the portal tracts.

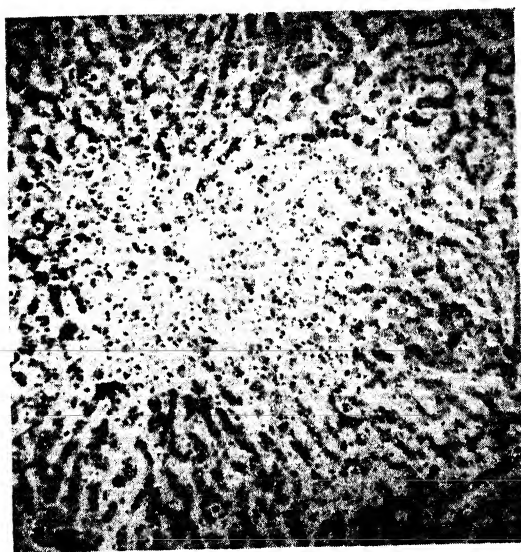


FIG. 7. Human. Fatal falciparum malaria.

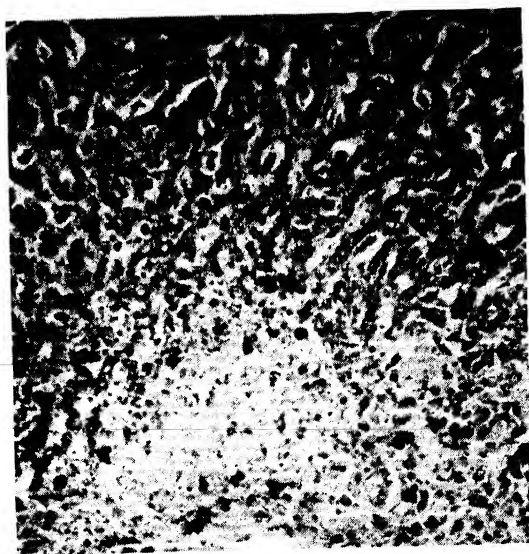


FIG. 8. Monkey. Fatal knowlesi malaria.

FIGS. 7 and 8 are included to emphasize the essentially different picture obtained in malaria.

chymal cells. If they do, the cells which are first in contact with the substance should show the most damage, as is seen after administration of the allyl formate or proteus toxin (Eppinger, 1937 ; Rosin and Doljanski, 1946).

The calibre of the liver sinusoids is diminished in carbon tetrachloride poisoning as a result of a swelling of the parenchymal cells. In places the degree of swelling is sufficient to obliterate the sinusoids almost entirely, and the consequent obstruction of blood-flow is sufficient to cause portal congestion. Other mechanisms obstructing the blood-flow through the liver lead to centrilobular changes, presumably due to anoxaemia, as has already been mentioned. We believe that the centrilobular necrosis seen after carbon tetrachloride and chloroform poisoning is also essentially due to anoxia of the hepatic cells, the anoxia in its turn being due to obstruction of the blood-flow through the sinusoids.

Mechanisms which cause swelling of the hepatic parenchymal cells are not understood, and further investigations are being made. As carbon tetrachloride is a general cytoplasmic poison, it is possible that the swelling may be due to a direct action on the cells, but this is still uncertain. Anoxia due to impedance of the blood in the hepatic veins usually leads to atrophy and degeneration (Chiari, 1899 ; Thompson and Turnbull, 1912), but on occasions hydropic necrosis may occur (Weatherford, 1935). Ischaemia of the sinusoids, presumably due to constriction of the efferent vessels to the lobule, has been noted by direct transillumination during inhalation of both carbon tetrachloride and chloroform poisoning (Wakim and Mann, 1942 ; Loeffler and Nordmann, 1925). It is therefore possible that the swelling of the parenchymal cells may itself be due initially to anoxia, and that, once the swelling has developed, anoxia of the central cells would be maintained by the changes in blood-flow.

The state of nutrition of the animal may also play a part in the reactions of parenchymal cells. The protective value of methionine and of carbohydrate is well known, the action probably being due to a modification of the swelling-response of parenchymal cells to carbon tetrachloride. Kosterlitz (1947) has stated that 'a fast of 24 hours will convert "hydropic degeneration" into "atrophy," a change which does not entail any further loss of liver cytoplasm.' A difference in diet may therefore account for the presence of hydropic necrosis in some rats and its absence in others after carbon tetrachloride administration, but in all animals swelling of the parenchymal cells was demonstrated.

Early congestion of the liver was noticed in two rats and in most of the guinea-pigs after administration of carbon tetrachloride. Cameron and Karunaratne (1936) found congestion more constantly present, especially in the central and mid-zones. It is difficult to see how an obstruction to the blood-flow through the sinusoids can lead directly to congestion and dilation of the central vein (fig. 2), and it is possible that this congestion is due to the initiation of the reflex postulated by Maegraith *et al.* (1947). Hepatic damage here might be the stimulus for the reflex constriction of the radicles of the hepatic vein, leading to an obstruction to the outflow of the blood. Engorgement may be present in the livers of patients dying after a therapeutic dose of carbon tetrachloride (Cameron and Karunaratne 1936), and in such cases there may be more than one mechanism leading to centrilobular anoxia.

SUMMARY

1. The hepatic lesion due to carbon tetrachloride poisoning in rats and other animals is described.

2. The lesion consists of centrilobular degeneration and necrosis. Swelling of the parenchymal cells arises.

3. The centrilobular nature of the lesion is ascribed to anoxaemia. Swelling of the parenchymal cells obstructs the flow of blood through the sinusoids and gives rise to centrilobular anoxia.

4. A method of outlining the hepatic sinusoids with Indian ink is given.

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INFECTION OF RETICULOCYTES BY *PLASMODIUM FALCIPARUM* AND *PLASMODIUM MALARIAE* IN HYPERENDEMIC INDIGENOUS MALARIA

BY

LEONARD JAN CHWATT

(From the Malaria Unit, Medical Department, Nigeria)

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The differential invasion of mature and immature red blood-cells by parasites of avian malaria was first observed by Ben-Harel (1923). It was later studied in human malaria by Eaton (1934), Jacobsthal (1936), Shushan, Blitz and Adams (1937), and more recently by Hingst (1938), Hegner (1938), and in particular by Kitchen (1938, 1939a, 1939b).

The earlier authors (Eaton and Jacobsthal) believed that *Plasmodium vivax* and *P. falciparum* infect immature red blood-cells by selection, and that *ipso facto* the majority of parasitized red blood-cells are reticulocytes.

Schüffner and de Graaf (1937) confirmed this opinion, but other authors (Malamos, 1937; Greig, 1934; Baserga, 1937) were less conclusive, since their results did not show any prevalent infection of reticulocytes by *P. falciparum*. Hegner (1938), however, reported that comparison of the relative frequency of infection of reticulocytes and mature blood-cells indicated that trophozoites of *P. falciparum* and of *P. malariae* seemed to favour immature red blood-cells. But Shushan *et al.* (1937) found that, while *P. vivax* showed a marked preference for immature red blood-cells, *P. falciparum* infected mature and immature cells with equal facility.

In his first paper (1938) Kitchen reported that *P. vivax* undoubtedly showed a greater tendency to invade reticulocytes.* This was confirmed by Vryonis (1939), who explained the higher rate of *vivax* infections of reticulocytes by the 'stickiness' of the immature cells.

A study of three cases of induced infection with *P. falciparum* and of two cases of infection with *P. malariae* led Kitchen (1939a) to the conclusion that *P. falciparum* is indifferent to the age of the red blood-cell—an opinion previously expressed by Shushan *et al.* (1937). It was also found by Kitchen that infections with *P. malariae* are seen almost exclusively in mature red blood-cells.

All the above investigations on the age of the red blood-cell infected with malaria parasites were carried out on, at most, two or three cases of natural or induced malaria.

* A recent paper by Ferrebee, Gibson and Peacock (1946) supports Kitchen's conclusion. Intravenous administration of a radio-active iron isotope, Fe 55, to a patient with benign tertian malaria was followed by an investigation of the comparative radio-activity of samples of whole blood and samples of a concentrate of infected red blood-cells. Radio-active iron, which is incorporated in the red blood-cells only during their formation, was found in samples of infected-cell concentrates in quantities 15–50 times as high as in samples of whole blood.

Although they enabled the investigators to follow up the trend of the infection of young and mature red blood-cells in relation to the clinical stage of the disease, they did not refer to persistent infections as seen in hyperimmune populations.

The present paper deals with the differential invasion of mature and immature erythrocytes in series of cases of indigenous subclinical malignant tertian and quartan malaria occurring in African children inhabiting a notoriously hyperendemic area.

TECHNIQUE OF THE INVESTIGATION

The investigation was carried out at Lagos, Nigeria, during the course of routine malaria surveys of the native child population between the ages of two and 10 years from the surrounding semi-rural areas.

The usual technique of investigation of parasite-rates adopted by the Nigerian Medical Department Malaria Unit consists of taking the thick drop and the thin film on the same slide, which, after the fixation of the thin film, is stained with one of the Romanowsky strains—preferably Giemsa.

For the investigation of the reticulocyte infection a second thin film, in addition to the routine blood slide, was taken from each child and stained with vital stain prior to counterstaining with Giemsa. Wright's stain, which was used by Kitchen for vital staining, was not available, and the excellent reticulocyte stain introduced by Laur (1932) under the name of vital violet (two parts of saturated solution of brilliant cresyl blue in absolute alcohol to one part of saturated solution of neutral red) was found less suitable for smears which had to be counterstained. The 'dry method' of reticulocyte staining was found to be more convenient for field conditions than Osgood's 'wet method,' which is based on mixing the blood-drop and the stain in a small tube before spreading on the slide. The 'dry method' gives slightly lower reticulocyte values, but its results are more consistent. Moreover, the 'dry method' was used by most of the previous investigators, and therefore enabled a better comparison of results to be made.*

The usual 'dry method' of vital staining was slightly modified for the purpose of the present investigation by depositing on one end of the slide a standard-sized (2 c.mm.) drop of a 1 per cent. solution of brilliant cresyl blue in 96° ethyl alcohol. After the alcohol had evaporated, the slide, with its dry blot of stain, was ready for use in the field, and could be stored easily and for indefinite periods. A drop of blood was placed on the dry blot of stain and thoroughly mixed for 15 seconds with the corner of another slide before being spread as a thin smear. Better results were obtained if the drying of the film was slowed down by breathing on the slide once or twice.

The technique of counterstaining was as follows. Thin films, vitally stained, were dried and treated for one minute with a 20 per cent. dilution of methyl alcohol in distilled water, to produce a partial fixation of the red cells. The slide was then immersed for

* It has been shown by Heath and Daland (1931), Davidson (1930), Laur (1932) and others that considerable differences in results of vital staining of reticulocytes may be due to the intervention of numerous physical and chemical factors in fixing and staining methods. Unduly long fixation of blood smears causes the fading of the vital staining, owing to the dissolving action of the alcohol on the reticulocyte substance; more concentrated stains produce coarser staining of the reticulum; glucose and sodium salts, high temperature and acid media inhibit staining, etc. All these factors may account for the surprising discrepancies in the results of similar investigations carried out by some of the authors cited.

30 minutes in a 1 : 20 solution of Giemsa in distilled water buffered to pH 7.2. The incompletely fixed red cells were thus considerably dehaemoglobinized and nearly transparent. The parasite seemed to be well fixed and stained deeply. The transparency of the red cells enabled the parasites to be picked out very easily and at the same time facilitated the counting of the reticulocytes, the deep blue network of which stood out very clearly.

This technique (without the use of any fixing agent) was described in 1907 by Chauffard and Fiessenger under the name of 'coloration hémolytique,' and was used for staining reticulocytes with Pappenheim's stain (Nicolle, 1936).

Of 340 positive blood samples examined during the spring of 1947, 61 were found suitable for detailed examination. Only those were selected in which the parasite species was identified and the parasite density was at least 1,000 per c.mm. for *P. falciparum* and at least 500 per c.mm. for *P. malariae*. Mixed infections were not included. The parasite density was estimated from the routine thick-drop slides, on the basis of the number of parasites per 400 leucocytes where the average white-blood-cell count in examined blood samples was known to be 8,000 per c.mm.

A total of approximately 50,000 red blood-cells, as represented by 100 fields each containing about 500 cells, was examined in each case, and the following data were recorded: (a) the number of infected mature red cells (normocytes); (b) the number of infected reticulocytes; (c) the number of non-infected reticulocytes. If, after examination of 100 fields, the number of reticulocytes counted was still less than 1,000, additional fields were searched until the 1,000 mark was reached. Only cells showing a definite reticulum, whether as clumps of coarse grains, or as a fine mesh, or as a sprinkle of fine grains, were counted as reticulocytes.

Apart from the parasite density per c.mm., the following rates were also calculated for each slide: (a) the number of infected normocytes per 100 red cells; (b) the number of infected reticulocytes per 100 reticulocytes; (c) the total number of reticulocytes per 100 red cells. The rate shown under (a) is obviously a function of the parasite density estimated from the thick drop. Results obtained from the two slides were then compared graphically and statistically, as a means of control of the counting technique. Their correlation appears to be satisfactory (see figs. 1 and 5, and Table IV).

The technique of counting *P. malariae* infections differed from that employed for *P. falciparum* in one respect: owing to the low density of the plasmodial infection, it was thought necessary to count at least 2,000 reticulocytes, instead of 1,000 as in the case of *P. falciparum*.

The amount of reticulocyte material present in infected immature red cells was recorded according to the classification proposed by Nicolle (1936), who recognizes three types of reticulocytes, 'rich,' 'medium' and 'poor'—the first being the most immature cell, with a compact lump of reticulum; the second, more mature, with coarse granules; the third having only a sprinkle of fine grains and being the nearest to a normal red blood-cell. This classification is simpler than that of Heilmeyer, who recognizes four types of reticulocytes.

RESULTS

In Tables I and II the results of the present investigation are tabulated separately for each species of malaria parasite and in order of increasing parasite densities.

The results of statistical analysis of the two series of observations are shown in Table III. The test compares the average infection-rate of normocytes and reticulocytes in individual cases in each parasite series. A different test—more justified in view of the fact that the infection-rates vary considerably from one case to another—can be applied

TABLE I
Differential infection of mature and immature red blood-cells with *P. falciparum*

Observation no.	Case no.	Parasite density per c.mm.	Reticulocytes per 100 fields = per 50,000 R.B.C.	Reticulocytes per 100 R.B.C.	Infected normocytes per 100 fields = 50,000 R.B.C.	Infected normocytes per cent.	Reticulocytes counted per 100 fields or at least 1,000 cells	No. of these reticulocytes infected	Infected reticulocytes per cent.
1	N 19	1,200	590	1.2	15	0.03	1,002	0	0
2	R 19	1,200	605	1.2	14	0.028	1,000	0	0
3	W 42	1,280	1,590	3.2	15	0.03	1,590	0	0
4	R 12	1,400	300	0.6	15	0.03	1,005	0	0
5	U 35	1,400	1,351	2.7	18	0.04	1,351	0	0
6	AA 17	1,420	1,702	3.4	20	0.04	1,702	0	0
7	AA 18	1,460	1,850	3.6	27	0.054	1,850	1	0.054
8	R 25	1,620	352	0.7	25	0.05	1,013	0	0
9	V 12	1,800	1,610	3.2	24	0.049	1,610	1	0.06
10	R 18	1,800	3,002	6.0	27	0.054	3,002	1	0.037
11	W 2	1,820	932	1.9	23	0.046	1,021	0	0
12	T 37	2,020	1,350	2.7	27	0.054	1,350	1	0.07
13	R 21	2,420	1,860	3.7	32	0.064	1,860	1	0.054
14	AA 30	2,420	1,503	3.0	33	0.066	1,503	0	0
15	N 30	2,460	1,490	3.0	29	0.058	1,490	1	0.074
16	V 23	2,660	2,803	5.6	34	0.068	2,803	2	0.07
17	V 23	2,800	902	1.8	35	0.07	1,003	1	0.1
18	U 19	2,840	1,592	3.2	41	0.08	1,592	1	0.063
19	R 52	2,840	620	1.2	42	0.084	1,002	1	0.1
20	N 29	3,240	1,263	2.5	44	0.088	1,263	1	0.08
21	V 5	3,280	3,100	6.2	38	0.076	3,100	2	0.065
22	T 18	3,420	1,001	2.0	40	0.08	1,001	2	0.2
23	T 23	4,200	1,022	2.0	56	0.11	1,022	2	0.2
24	T 27	4,800	3,005	6.0	58	0.12	3,005	3	0.09
25	U 23	5,200	1,240	2.4	70	0.14	1,240	0	0
26	AA 27	5,600	2,570	5.2	67	0.13	2,570	1	0.08
27	R 27	6,000	1,257	2.5	75	0.15	1,257	1	0.08
28	U 3	6,200	2,420	4.9	68	0.136	2,420	1	0.04
29	T 12	6,200	376	0.8	79	0.16	1,005	1	0.1
30	W 7	6,260	2,150	4.3	82	0.164	2,150	4	0.2
31	U 17	7,260	2,820	5.6	79	0.158	2,820	2	0.07
32	W 40	7,280	2,512	5.0	92	0.185	2,512	3	0.24
33	R 28	8,400	2,314	4.6	119	0.24	2,314	16	0.7
34	U 32	8,460	5,166	8.0	111	0.22	5,166	4	0.1
35	V 39	9,400	2,716	5.4	108	0.2	2,716	6	0.22
36	AA 2	10,200	4,450	8.3	132	0.26	4,450	6	0.14
37	N 16	12,040	2,493	5.0	203	0.4	2,493	9	0.36
38	U 27	12,600	3,304	6.6	191	0.39	3,304	5	0.22
39	O 23	13,000	3,040	6.0	182	0.36	3,040	8	0.260
40	O 15	14,100	3,052	6.1	175	0.35	3,052	4	0.13
41	O 9	16,200	6,250	12.5	261	0.52	6,250	31	0.495
42	N 10	20,200	2,900	5.8	275	0.55	2,900	14	0.49
43	T 51	22,100	5,908	11.8	385	0.76	5,928	42	0.84
44	T 42	32,600	6,344	12.6	505	1.0	6,244	34	0.54
45	W 31	56,200	5,842	11.7	805	1.6	5,912	46	0.78

TABLE II

Differential infection of mature and immature red blood-cells with *P. malariae*

Observation no.	Case no.	Parasite density per c.mm.	Reticulocytes per 100 fields = per 50,000 R.B.C.	Reticulocytes per 100 R.B.C.	Infected normocytes per 100 fields = 50,000 R.B.C.	Infected normocytes per cent.	Reticulocytes counted per 100 fields or at least 2,000 cells	No. of these reticulocytes infected	Infected reticulocytes per cent.
1	U 12	520	621	1.2	6	0.012	2,000	0	0
2	R 20	620	1,214	2.4	8	0.016	2,002	0	0
3	R 31	660	1,010	2.0	7	0.014	2,007	0	0
4	T 39	660	1,528	3.0	10	0.020	2,003	0	0
5	V 19	680	1,243	2.5	7	0.014	2,003	1	0.05
6	W 17	720	1,740	3.4	9	0.018	2,011	0	0
7	TT 20	780	1,075	2.2	9	0.018	2,010	0	0
8	T 25	800	1,758	3.5	8	0.016	2,003	0	0
9	V 31	860	1,004	2.0	12	0.024	2,004	0	0
10	U 24	920	4,075	8.2	10	0.02	4,075	1	0.024
11	T 10	1,200	1,420	2.8	14	0.028	2,000	0	0
12	U 2	1,480	1,961	3.8	18	0.036	2,005	0	0
13	Z 2	1,860	2,982	6.0	22	0.042	2,982	1	0.032
14	K 12	2,800	4,087	8.2	38	0.076	4,087	1	0.024
15	M 9	3,600	2,682	4.6	45	0.09	2,682	0	0
16	B 17	4,200	3,348	6.6	63	0.126	3,348	1	0.03

TABLE III

Statistical analysis of series of infections with *P. falciparum* and *P. malariae*

No. of observations	<i>P. falciparum</i>		<i>P. malariae</i>	
	45		16	
	Infected normocytes per cent.	Infected reticulocytes per cent.	Infected normocytes per cent.	Infected reticulocytes per cent.
Mean of whole series ...	0.21	0.15	0.036	0.01
Standard deviation of mean	0.043	0.039	0.008	0.004
Standard error of mean ...	±0.086	±0.078	±0.016	±0.008
Standard error of the difference between the two means	0.058		0.01	
Significance	The difference between the two means is 0.06, or almost equal to the standard error of the difference, and therefore not significant		The difference between the two means is 0.025, or 2.5 times the standard error of the difference, and therefore significant	

The data for *P. malariae* were corrected for small series of observations.

to over-all differences observed in the infection-rate of the total number of normocytes and reticulocytes in each series (Table IV).

TABLE IV

Statistical analysis of over-all infections of normocytes and reticulocytes in *P. falciparum* and *P. malariae* series

	<i>P. falciparum</i>		<i>P. malariae</i>	
	Normocytes	Reticulocytes	Normocytes	Reticulocytes
No. of cells examined ...	2,250,000	107,883	800,000	39,222
No. of infected cells ...	4,796	260	286	5
Proportion of infected cells	0.002132	0.00241	0.0003575	0.000125
Standard error ...	0.000031	0.00015	0.000021	0.000056
Standard error of the difference ...	0.000153		0.0000598	
Significance ...	The standard error of the difference is 1.817 times the standard error; not significant		The standard error of the difference is 3.888 times the standard error; very significant	

Comparison of the relative frequency of observations of infected mature and immature erythrocytes and of the intensity of the infection (Kitchen, 1939b) gives the results shown in Table V.

TABLE V

Comparative incidence and intensity of infection in *P. falciparum* and *P. malariae* series

	Relative incidence of infection			Relative intensity of infection					
	Normocytes more often than reticulocytes	Reticulocytes more often than normocytes	No difference	Normocytes			Reticulocytes		
				Maximum	Minimum	Mean	Maximum	Minimum	Mean
<i>P. falciparum</i> series	31 observations 69%	13 observations 29%	1 observation 2%	1.6	0.028	0.21	0.084	0	0.15
<i>P. malariae</i> series	13 observations 82%	3 observations 18%	Nil	0.126	0.012	0.036	0.05	0	0.01

Figures referring to intensity are percentages of infected cells.

Scatter-diagrams were plotted for each series of infections, to show the relationship between the following pairs of characteristics.

Figs. 1 and 5: parasite density per c.mm. versus the number of infected normocytes per 100 red blood-cells (these diagrams and their correlation coefficient serve as a control of the counting technique).

Figs. 2 and 6: parasite density per c.mm. versus the number of infected reticulocytes per 100 reticulocytes.

Figs. 3 and 7 : parasite density per c.mm. versus the total number of reticulocytes per 100 red blood-cells (degree of reticulocytic response).

Figs. 4 and 8 : total number of reticulocytes per 100 red blood-cells versus the number of infected reticulocytes per 100 reticulocytes.

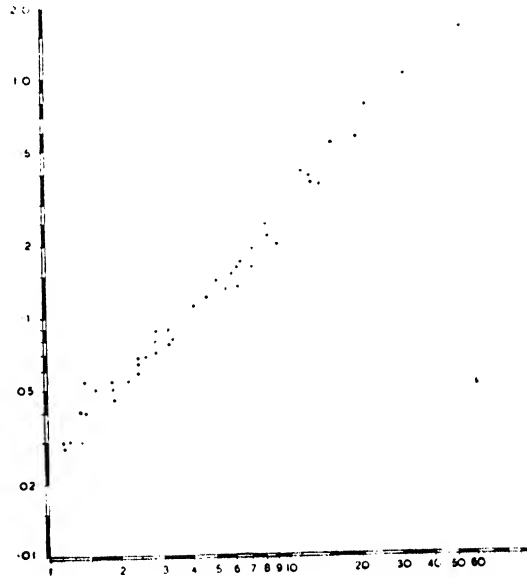


FIG. 1. *P. falciparum* series. x = Parasite density, in thousands per c.mm. ; y = Infected normocytes per 100 red blood-cells. Log/log grid.

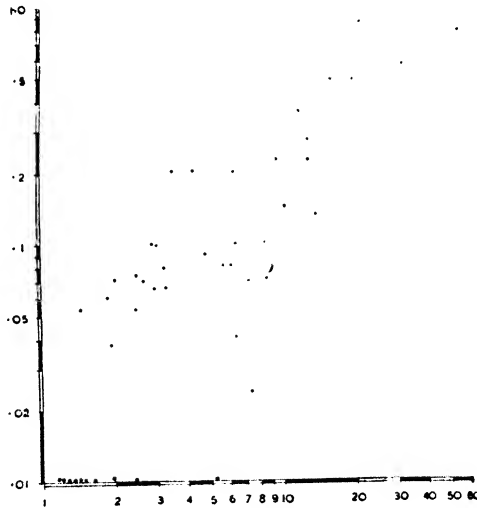


FIG. 2. *P. falciparum* series. x = Parasite density, in thousands per c.mm. ; y = Infected reticulocytes per 100 reticulocytes. Log/log grid.

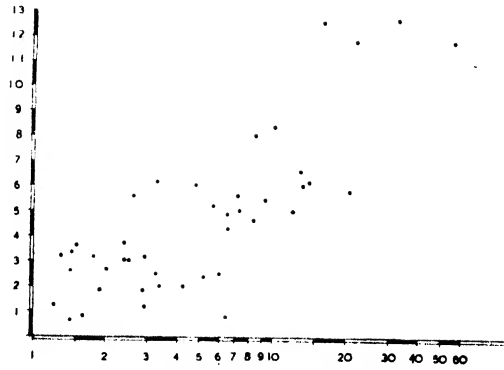


FIG. 3. *P. falciparum* series. x = Parasite density, in thousands per c.mm. ; y = Reticulocytes per 100 red blood-cells. Arithlog grid.

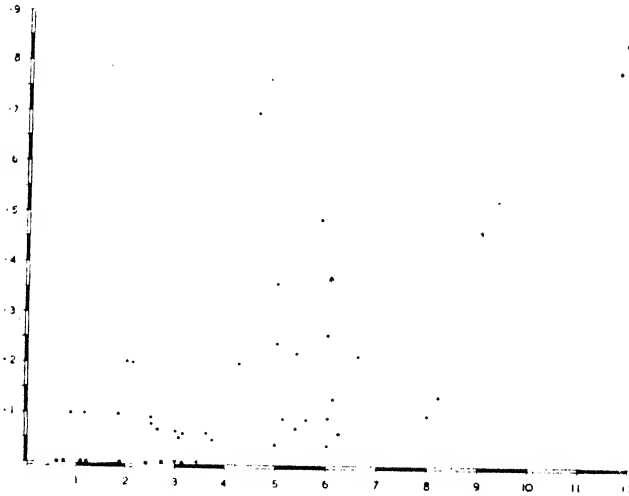


FIG. 4. *P. falciparum* series. x = Reticulocytes per 100 red blood-cells ; y = Infected reticulocytes per 100 reticulocytes. Arithmetic grid.

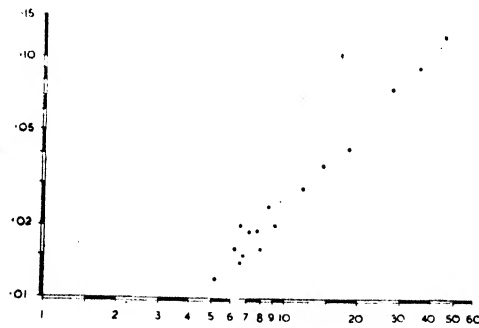


FIG. 5. *P. malariae* series. x = Parasite density, in hundreds per c.mm. ; y = Infected normocytes per 100 red blood-cells. Log/log grid.

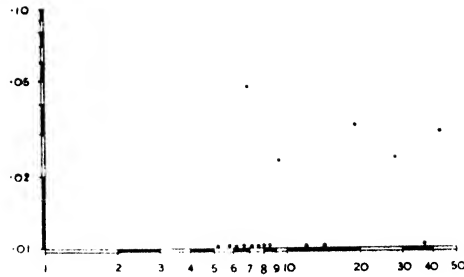


FIG. 6. *P. malariae* series. x = Parasite density, in hundreds per c.mm.; y = Infected reticulocytes per 100 reticulocytes. Log/log grid.

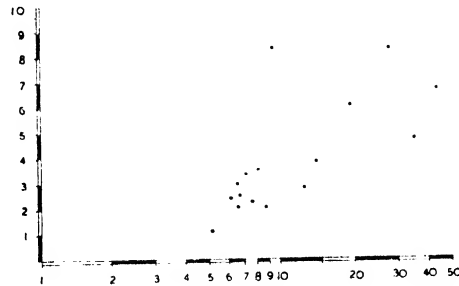


FIG. 7. *P. malariae* series. x = Parasite density, in hundreds per c.mm.; y = Reticulocytes per 100 red blood-cells. Arithlog grid.

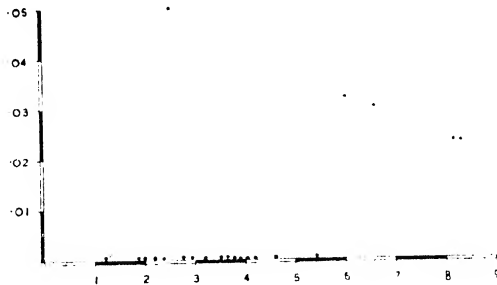


FIG. 8. *P. malariae* series. x = Reticulocytes per 100 red blood-cells; y = Infected reticulocytes per 100 reticulocytes. Arithmetic grid.

The respective correlation coefficients, expressing the degree of association between each pair of characteristics, together with their tests of significance, are shown in Table VI.

In Table VII are shown the results of the investigation of the relation of the incidence of the infection of immature red cells to the amount of their reticulocyte material.

Variations of the parasite density in *P. falciparum* infections and of the degree of reticulocytosis were investigated in five children aged between five and seven years. Repeated examinations were carried out at intervals of 14 days. The results of this investigation are shown in Table VIII.

TABLE VI
Comparative value of correlation coefficients

Pair of characteristics	<i>P. falciparum</i> series	<i>P. malariae</i> series	Figs.
Parasite density versus infected normocytes	+0.92	+0.89	1 and 5
Parasite density versus infected reticulocytes	+0.61	+0.2	2 and 6
Parasite density versus reticulocytes per 100 red blood-cells	+0.87	+0.56	3 and 7
Reticulocytes per 100 red blood-cells versus infected reticulocytes	+0.56	+0.33	4 and 8
Standard error of the correlation coefficient	0.15	0.25	
Test of significance	All coefficients unlikely to have arisen by chance	Second and fourth coefficients likely to have arisen by chance	

TABLE VII
Infection of immature red blood-cells containing varying amounts of reticulocyte material

	Type of infected reticulocyte		
	Rich	Medium	Poor
<i>P. falciparum</i> series	12 34%	15 43%	8 23%
<i>P. malariae</i> series	2 40%	1 40%	2 20%

TABLE VIII
Variations of parasite densities and of the degree of reticulocytosis in five selected cases of infection with *P. falciparum*

Case no.	1st examination		2nd examination		3rd examination		4th examination	
	Parasite density per c.mm.	Reticulo-cytes per cent.	Parasite density per c.mm.	Reticulo-cytes per cent.	Parasite density per c.mm.	Reticulo-cytes per cent.	Parasite density per c.mm.	Reticulo-cytes per cent.
R 28	8,400	4.6	6,200	3.8	6,000	4.8	—	—
AA 2	10,200	8.3	7,800	8.0	7,200	6.8	—	—
U 27	12,600	6.6	14,200	10.2	12,000	10.8	12,600	12.4
O 9	16,200	12.5	8,600	8.2	10,800	8.4	—	—
N 10	20,200	5.8	16,600	10.4	8,200	6.8	6,400	8.5

DISCUSSION

The clinical and parasitological material used in the present investigation consisted of series of cases of chronic untreated malaria in children from a hyperendemic area of Nigeria. This material is entirely different from that used by Kitchen and other investigators, who examined small numbers of cases of natural or—more often—induced malaria. Nevertheless, the results reported here corroborate the findings of Shushan *et al.* and, more particularly, of Kitchen.

It appears that *P. falciparum* does not show any marked preference for immature red blood-cells, but that it invades both reticulocytes and normocytes more or less indiscriminately. On the other hand, *P. malariae* seems to invade immature red cells only in exceptional cases.

Statistical analysis of the results of the investigation appear to justify the stressing of the following points.

1. *P. falciparum* series. (a) There is no statistically significant difference between the mean rate of infected normocytes and the mean rate of infected reticulocytes in a series of 45 individual cases; observations of infected reticulocytes might be ascribed to chance distribution. (b) The same conclusion applies to findings based on the statistical analysis of over-all infections of red cells in the whole series.

2. *P. malariae* series. (a) The difference between the mean rate of infected normocytes and the mean rate of infected reticulocytes in a series of 16 individual cases is statistically significant and cannot be ascribed to chance distribution. (b) This conclusion is emphasized by analysis of over-all infections of red blood-cells in the whole series.

Young reticulocytes do not seem to be invaded by *P. falciparum* more frequently than older reticulocytes.

A study of the scatter-diagrams and their correlation coefficients shows that in the case of *P. falciparum* infections the proportion of infected reticulocytes is related to the parasite density. There is no such relationship in the *P. malariae* series. In the case of *P. falciparum* the high parasite density produces high reticulocyte response. This is much less obvious in the *P. malariae* series. It must be pointed out, however, that densities above 1,500 quartan parasites per c.mm. were rarely observed.

Closer study of the relationship between *P. falciparum* density and the degree of resulting reticulocytosis shows that in *P. falciparum* infections of relatively low density (below 5,000 parasites per c.mm.) the average reticulocyte response is 3.6 per cent. The average degree of reticulocytosis found in 21 African children of the same age-group whose blood slides were repeatedly negative was found to be 2.4 per cent. Thus it seems that a relatively mild *P. falciparum* infection in hyperimmune children is followed by a reticulocyte response slightly above the values seen in children without any evidence of malarial infection.

High and very high degrees of reticulocytosis are nearly always observed in chronic, though subclinical, *P. falciparum* infections, where the density varies between 10,000 and 60,000 parasites per c.mm.

Repeated examinations of the peripheral blood of five children with high *P. falciparum* parasitaemia, but without any clinical symptoms of malaria, have shown that the degree of reticulocytosis generally varies in relation to the varying level of parasite densities. The variations of the reticulocyte response were, on the whole, less marked than the corresponding degree of the parasitaemia.

Reticulocyte response as a sign of blood regeneration in malaria was observed in induced infections following the administration of antimalarial drugs. Fairley (1934) found that a maximum response is proportional to the degree of anaemia.

It seems that, in African children from a hyperendemic area, a persisting, untreated, subclinical malaria infection with *P. falciparum* produces a state of prolonged increased erythropoiesis, due to the existence of a haemolytic stimulus and related to its severity.

SUMMARY

The differential infection of mature and immature red blood-cells by *Plasmodium falciparum* and *P. malariae* was investigated in 61 cases of malaria in African children from two to 10 years of age from a hyperendemic area in the coastal belt of Nigeria.

A modified method of 'haemolytic staining' of parasitized reticulocytes was used with satisfactory results.

It was found that *P. falciparum* invades immature red cells about as often as mature cells, and that the occurrence of infected reticulocytes may be due to chance distribution.

P. malariae invades immature red cells only in exceptional cases, and statistical analysis suggests that this parasite prefers the mature red cell.

Untreated *P. falciparum* malaria in hyperimmune African children produces a prolonged reticulocyte response. The degree of this response is related to the parasite density.

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DISCUSSION OF METHODS FOR DIFFERENTIATING TICK- FROM LOUSE-BORNE RELAPSING FEVER SPIROCHAETES

BY

N. F. COGHILL

(*Senior Physician, West Middlesex County Hospital.
Late Major, R.A.M.C., Medical Specialist*)

AND

R. M. GAMBLES

(*Veterinary Research Officer, Government of Cyprus*)

(*Received for publication January 19th, 1948*)

During recent studies of relapsing fever (to be published later), in particular in an attempt to arrive at a more accurate clinical differentiation of the two types, we were struck, when studying the literature of this aspect of the disease, by the frequent absence of reliable evidence as to the vector. If the vector is not known, descriptions of symptoms and signs lose value, and any attempt to assess the differences and similarities of the two diseases is hampered. In such a comparative study this point is of considerable importance, for various workers have noted that the manifestations, perhaps especially in the tick-borne form, are often relatively few, and in uncomplicated cases may be no more than those associated with any common fever, such as influenza, malaria, sandfly-fever or dengue. It is usually considered that differentiation of the two types can seldom be made on clinical grounds alone. Although this is often so in the individual case, it is probably less true with larger numbers. An easy laboratory method of separating them would undoubtedly assist in clarifying their clinical aspects. Most of such methods are lengthy and in general unreliable, but guinea-pig inoculation is held by a number of workers to be satisfactory. The usefulness and general applicability of this simple biological test does not yet seem to be as widely known as it deserves. It has important bearings on the epidemiological and clinical aspects of relapsing fever.

Guinea-pigs are stated to be naturally almost completely immune to louse-borne spirochaetes and always, or nearly always, to react to the tick-borne strains (Kirk, 1938; *Tropical Diseases Bulletin*, 1938; Adler and Ashbel, 1942; Dixon, 1943). Although this is in large part true, the division may not always be so decisively clear-cut.

Against these opinions we find the following statements. Wenyon (1926) writes: 'It has been claimed that animals are more readily infected with *T. duttoni* than other supposed species, but there is no real ground for this assertion. Individual strains of one and the same species vary considerably in their virulence for animals.' Knowles (1928) makes no mention of guinea-pigs under his heading 'Susceptibility of Animals' but quotes from Wenyon (1926) as follows: 'Leishman (1907) stated that he could find no morphological difference between the European, Central African and Indian strains, and that the supposed differences in virulence for laboratory animals did not exist, . . .

The various other distinguishing features which have been adduced, such as . . . the variations in susceptibility of laboratory animals to inoculation cannot be regarded as reliable methods of differentiating species.' Rogers and Megaw (1944) do not mention guinea-pigs, but, writing of the tick-borne form, they state: 'The spirochaete can be inoculated directly into rabbits, mice, dogs and other animals which are only susceptible to infection by the *Spirochaeta recurrentis* after passage through monkeys, etc.' Manson-Bahr (1945) mentions guinea-pigs only in relation to the relapsing fever of south Spain and Morocco, south Russia, Persia, Palestine and north-west India, stating that the animal is susceptible to these strains. Strong (1945), without distinction of types, writes: 'Guinea-pigs are usually resistant to infection with many strains.' Topley and Wilson (1946), making no distinction between louse- and tick-borne types, state that 'Infection can be transmitted to monkeys, rats and mice and, with some types of spirochaetes, to guinea-pigs.'

Much work has been done on this subject. Sawtschenko and Melkich (1901) established the natural immunity of the guinea-pig to *Sp. obermeieri* (syn. *recurrentis*). Norris *et al.* (1906) and Novy and Knapp (1906) could not infect guinea-pigs with spirochaetes from a case of presumed louse-borne relapsing fever of Carlisle (1906). Moskwins (1929) found guinea-pigs susceptible to a tick-borne strain of Russian relapsing fever. Remlinger and Bailly (1929) confirmed previous work of Nicolle and Anderson in showing the guinea-pig to be invariably susceptible to *Sp. hispanicum* var. *maroccanum*. Monti (1930) in experiments with *Sp. hispanicum* found the guinea-pig to be the most receptive animal. Hindle (1931) discusses the pathogenicity of various strains of spirochaete for different animals, and, while on the one hand he mentions that occasionally *Sp. recurrentis* may be directly inoculable from man to guinea-pig, on the other he states that these animals are refractory to two South American tick-borne strains—*Sp. venezuelensis* and *Sp. neotropicalis*. Nicolle (1932), as the result of extensive work, writes as follows (the translation is ours): 'The diagnostic animal is the guinea-pig. Immune, or almost immune to the louse-borne infection, it contracts, following the inoculation of the Spanish-African strain a true relapsing fever, characterized by febrile attacks, in the course of which the spirochaetes are numerous in the blood.' But this strain is only one of the tick-borne spirochaetes in North Africa, and Nicolle goes on to discuss *Sp. normandi*, transmitted by *Ornithodoros normandi*, which he found hardly pathogenic to man and non-pathogenic for guinea-pigs. Sergeant (1933) and Sergeant *et al.*, (1933, 1935) describe three cases of relapsing fever transmitted by *Rhipicephalus sanguineus* in Algeria; the spirochaetes of all these cases were inoculable into guinea-pigs. Kemp *et al.* (1934), using tick-borne strains of Texan relapsing fever, state: 'We failed to produce infections uniformly in guinea pigs either by intraperitoneal or intravenous inoculation of heavily infected onset blood taken from rats.' Coleman (1934), working with Californian tick-borne spirochaetes, found guinea-pigs variably susceptible, but, although their blood might remain microscopically negative, it was always infectious for mice. Brumpt (1935), who has worked extensively with relapsing fever, speaks of the differentiation of strains by guinea-pig inoculation as if it were an established fact. Aluimov (1935) writes in the same vein. Working with a strain of *Sp. persica*, Adler and Ashbel (1937) found that only two guinea-pigs out of 290 were refractory, but that in five more the infection was slight and transient, although leaving a residual brain infection. The same authors (1942) later stated: 'As far as Palestine is concerned, infectivity for guinea-pigs can be recommended as a simple practical

test for distinguishing between tick- and louse-borne spirochaetes. Wherever *Spirochaeta obermeieri* has been examined it has been found non-infective for guinea-pigs, whereas, out of 42 strains acquired in Palestine and Syria, 40 were found to produce heavy infections in guinea-pigs, 1 was only slightly infective . . . and 1 was practically non-infective . . . Baltazard (1937) isolated strains of spirochaete from *O. erraticus* in south Morocco, which, although highly pathogenic to rats, produced only a non-apparent infection in guinea-pigs. Davis (1939) found that 10 different strains from three species of American ticks were all inoculable into guinea-pigs. Pospelova-Shtrom (1940) and Sofiev (1941) failed to infect guinea-pigs with *Sp. latyschewi* isolated from *O. tartakowskyi*. Sergeant and Richard (1942) found only one guinea-pig out of 3,190 completely immune to a strain of Spanish-North African spirochaete. Birkhaug (1942) comments on the fact that Obermeier completely failed to produce the picture of relapsing fever in dogs, rabbits and guinea-pigs by inoculation of infected human blood. Bohls and Irons (1942) discuss animal susceptibility with regard to New World tick-borne spirochaetes and note that different strains vary in their inoculability into guinea-pigs. Sautet (1941), working with a spirochaete transmitted by *O. tholozani* near Damascus, found guinea-pigs developing a severe infection with it. Wolman and Wolman (1945) found that guinea-pigs were naturally resistant to a strain of Abyssinian *Sp. recurrentis* (louse-borne) but that they could be made relatively susceptible by blocking the reticulo-endothelial system. Guinea-pigs were found by Baltazard (1946) to be almost completely insusceptible to a louse-borne strain of spirochaetes in Iran.

It seems clear that louse-borne relapsing fever spirochaetes can very seldom infect guinea-pigs on direct inoculation. It appears, however, that some tick-borne strains are also non-pathogenic for these animals. This is a commoner finding in the New World than in the Old. At other times tick-borne strains give rise to such a mild infection in guinea-pigs that spirochaetes are never detectable in the peripheral blood on microscopic examination, although either the blood or the brain in these cases will usually be infectious for mice or rats. Louse-borne strains do not behave in this manner, and almost invariably have to be passed through monkeys before becoming inoculable into guinea-pigs.

The most important point of this biological test is that if any given strain of relapsing fever spirochaetes is found to be infectious for guinea-pigs it must be tick-borne in origin. If it is seemingly non-infectious, attempts must be made to exclude a 'non-apparent' infection by subpassage through rats or mice and by using larger numbers of guinea-pigs. Even if this meets with negative results the certainty of the strain's being louse-borne is not complete, although it is considerably increased.

If there is difficulty over the use of guinea-pigs, mice and rats are fairly good substitutes, being more susceptible to tick-borne spirochaetes than to louse-borne on direct inoculation. With louse-borne spirochaetes the disease is milder and runs a shorter course. Strains can be tested for guinea-pig inoculability via rats or mice instead of being done direct—a manoeuvre which may save time and guinea-pigs and may help to show up the infection. Rats and mice may certainly be of more use in routine searches for spirochaetes in ticks.

The minor fallacies of the guinea-pig inoculation test, therefore, although they exist, are probably insufficient to detract materially from its value in practice, at least in Europe, Africa and Asia, where many of the tick-borne strains of spirochaetes which are non-pathogenic for guinea-pigs are also non-pathogenic, or almost so, for man.

SUMMARY

A review is given of the literature bearing on the biological test of guinea-pig inoculation for differentiation of tick- from louse-borne strains of relapsing fever. The following conclusions seem permissible:

1. If any strain of relapsing fever spirochaete is infectious for guinea-pigs it is highly probable that it is tick-borne in origin.
2. It is exceedingly rare for louse-borne strains to be directly inoculable into guinea-pigs.
3. Some strains of tick-borne spirochaetes may produce only non-apparent infections in guinea-pigs, and a few may be completely non-pathogenic to these animals.

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MISCELLANEA

THE OCCURRENCE OF A PIROPLASM, *ENTOPOLYPOIDES MACACI*, IN EAST AFRICAN MONKEYS

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Large numbers of monkeys, *Cercopithecus aethiops*, are used in Tinde laboratory, and during the examination of their blood many were found to be infected with *Plasmodium kochi*. A few, however, were infected with another parasite, which was not identified. Thin blood films, made from monkey 489 in July, 1943, and from monkey 609 in May, 1944, were submitted to Dr. C. M. Wenyon, who stated that the organism was a piroplasm, known as *Entopolypoides macaci* Mayer, 1933.

Mayer (1933, 1934) found this piroplasm in the blood of two monkeys, *Macaca irus*, from Java, and described it fully when establishing it in a new genus and species. His second paper is illustrated with a coloured plate of the various forms of the parasite. The organism is very different from *Babesia pitheci*, the usual piroplasm of *Cercopithecus* monkeys; and the present record is the first instance of *E. macaci* being found either in Africa or in a species of *Cercopithecus* monkeys. Mayer stated that even when the infection was a heavy one it seemed to have little effect on the health of its host, and that has also been our experience.

I am very grateful to Dr. Wenyon for identifying this parasite and referring me to the literature, and to Dr. C. A. Hoare for his help.

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H. FAIRBAIRN,
Tinde Laboratory,
Tanganyika Territory.

THE EFFECT OF CERTAIN INORGANIC AND VEGETABLE SUBSTANCES ON THE ENGLISH POND SNAIL *PLANORBIS CORNEUS* (LINNÉ, 1758)

BY

S. G. COWPER

(From the Department of Parasitology, London School of Hygiene and Tropical Medicine)

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I. REVIEW OF THE LITERATURE AND EXPERIMENTS ON THE ACTION OF COPPER

INTRODUCTORY REMARKS AND REVIEW OF THE LITERATURE

Copper has long occupied a unique position as a molluscicide. Its toxic action against freshwater snails and green algae, together with its comparative harmlessness to higher vertebrates and phanerogamic vegetation, have, when considered in conjunction with its relative inexpensiveness, made it the most favoured agent for snail-control campaigns.

A voluminous literature has appeared on the efficacy of various copper preparations—of which copper sulphate is the most popular—when tested against snails both *in vitro* and in the field, and large-scale snail-destruction campaigns, sometimes combining the use of copper with periodic drying, have been carried out in Egypt, Southern Rhodesia and elsewhere, as part of bilharzia-control schemes.

Khalil (1932) has given a detailed account, based on an extensive series of experiments in Egypt, of the value and limitations of copper as a snail-killer, and the developments in its use, which have been going on since Leiper's classical researches, are described in numerous publications by Barlow and Abdel Azim and others, who have established the real value of 5 parts per million of copper sulphate as a means of control in the field.

Various inorganic chemicals other than copper have also been tried. The pioneer Japanese investigators of Far Eastern bilharziasis, whose work is summarized by Faust and Meleney (1924), experimented with lime, calcium cyanamide, ammonium sulphate, calcium phosphate and bleaching-powder, all of which were employed with some success; and Leiper (1915) recommended that the periodic drying of irrigation canals and ditches should be accompanied by the addition of ammonium sulphate to the small residual pools. More recently, Mozley (1944), working in Southern Rhodesia, has investigated the molluscicidal action of certain other inorganic compounds, viz., barium-silico-fluoride, zinc formaldehyde sulphonylate, silver mercuric iodide and silver metal colloidal, as well as the use of crude copper ores, especially malachite. The possible value of the new synthetic insecticide 'Gammexane' as a snail-killer has also been investigated (Halawani, 1946, 1947).

Vegetable molluscicides offer a possible alternative to the use of copper in places where the expense or difficulty of obtaining copper salts or ores or of their transportation is great, and in this connection attention has recently been directed to the extracts of certain tropical plants, many of which are traditionally used by native races as fish poisons.

Mozley (1944) investigated the value of a number of these plants in Southern Rhodesia, while Floch and Lajudie (1946) have noted the snail-killing properties of a saponin present in *Sapindus saponaria* in French Guiana.

The object is to break the bilharzial cycle by destroying the vector; and, while the final tests for suitable agents, concentrations, technique, etc., must be carried out in the field, useful and interesting information can be deduced from experiments carried out in the laboratory on snails, tropical or temperate.

The work described here concerns investigation or confirmation of the relative efficacy of molluscicidal agents, including inorganic chemicals and extracts of certain tropical plants, on a standard British snail, *Planorbis corneus* (Linné, 1758), the 'black *Planorbis*' of English ponds, which is easily maintained under laboratory conditions.

In certain experiments one or two specimens of the more delicate tropical American species *Australorbis glabratus* were added to the *P. corneus*, no particular difference in the reaction of the two species to various agents being noted.

It is recognized that the value of the experiments is diminished by the small number of snails used (five or 10 in each exposure). The limited number of snails available and the slow rate at which they breed in aquaria made this unavoidable. But, since most dilutions of the various test-agents killed or rendered prostrate either all or none of the exposed snails, results are probably significant.

The present paper deals first with experiments using copper salts and ores. Ordinary tap-water (the pH of London tap-water is about 8) was used as a diluent, except in making up the dilutions of copper salts and in some of the saponin experiments, where distilled water was used.

Throughout this paper p.p.m. = parts per million, and 'reacts to touch' implies that the snail, though apparently lifeless, shows a reaction when touched with a pointed instrument.

EXPERIMENTS WITH COPPER SALTS

The agents tested consisted of certain copper salts (ordinary bench reagents), ground-down copper ores and metallic copper. Leiper (1921) states that electrolytically produced salts are more toxic to snails than ordinary bench reagents. Those used in our experiments were electrolytes in solution but were not electrolytically prepared.

Khalil (1932) makes certain generalizations, based on work in Egypt, concerning the use and action of copper, and notes that the higher plants, most vertebrates—including man—and certain invertebrate groups (e.g., Crustacea, Oligochaeta) are resistant, whereas the lower plants, molluscs and certain species of fish and frogs are very susceptible, as are also mosquito larvae and protozoa. There seems to be little danger, therefore, of any toxic effects on man, domestic animals or germinating crops, so long as the copper is used only in the proportions necessary for snail control.

In the Dakhla oasis, Khalil and Abdel Azim (1938) found that sulphation (i.e., treatment with CuSO_4) at 5 p.p.m. caused disappearance of the snails within five days. Barlow and Abdel Azim (1945) found that two successive applications of CuSO_4 at 7 p.p.m. left to act for 48 hours and five days respectively gave a 99 per cent. kill. They noted the steady fall in concentration due to decomposition of the copper or its combination with algae, and recommended higher concentrations of copper and weed clearance preceding sulphation. The same authors (1946) give the results of a wide survey following sulphation

at 15–30 p.p.m., showing reductions in numbers of *Buunus* from 63 to 37, and of *Planorbis* from 125 to 5, per 100 dips.

Chandler (1920), working on American snails of various species, found that of various inorganic chemicals tested only mercuric chloride was comparable in efficiency to copper, and this salt is more expensive. He found that CuSO_4 might be effective against algae at a dilution as high as 1 : 25,000,000. Bacteria were destroyed at 2.5 p.p.m., and protozoa at 3–15 p.p.m. Fish were susceptible to 1–2 p.p.m. All the species of snail used died within 48 hours at 1–2 p.p.m., although copper has no effect on snail eggs. The action of copper is influenced by four extrinsic factors, viz., temperature, presence of algae, alkalinity and organic matter in solution.

Mozley (1944) describes a very comprehensive series of experiments with copper preparations (copper sulphate, copper acetate, copper chloride), and records an appreciable kill (*in vitro*) at such low dilutions as 0.1 p.p.m. He finds that the copper ion, not the acid radicle, is the toxic agent. Malachite (mineralized copper carbonate) gave a 100 per cent. kill at 400 p.p.m. in 18 hours or at 50 p.p.m. in 48 hours (*Biomphalaria*), and at 400 p.p.m. in 24 hours (*Physopsis*). His report gives full details of methods of application of copper in the field and recommends the large-scale use of malachite mixture consisting of copper carbonate, sawdust and powdered pods of the plant *Swartzia madagascariensis*. In the Southern Rhodesian Government's public health report for 1945, it is pointed out that the malachite mixture requires 2,500 p.p.m. to give the same effect as is given by copper sulphate at 8 p.p.m., and that copper sulphate is 100 times cheaper and 300 times less weight for transport. The above refers to species of *Bulinus*, *Planorbis* (= *Biomphalaria*) or *Physopsis*.

Buxton and Krikorian (1922), working in Palestine, compared the action of Fowler's solution, potassium cyanide, oxalic acid, potassium bichromate, sodium arsenite and liquor cresolis on *Melanopsis puerosa* and *Bulinus truncatus*, and found that none of these was equal to copper sulphate, which killed snails after 72 hours' exposure to 1 p.p.m.

The present experiments, carried out in the laboratory with copper salts and *P. corneus*, gave the results shown in Table I.

Comparing these results with those recorded by Mozley, Khalil, Abdel Azim and others, it seems that *P. corneus*, under the conditions recorded, shows greater resistance to copper than do *Bulinus*, *Physopsis* and other tropical snails. The sulphate, the acetate and the chloride salts of copper are all effective. Copper acetate killed all five snails at 1 p.p.m. in 48 hours. Copper chloride killed all five at 5 p.p.m. in 24 hours. Copper carbonate, which is much less soluble than the other salts, is toxic at 5 p.p.m.

EXPERIMENTS WITH COPPER ORES

It has been suggested that the use of ground-up crude copper ores may be of value in snail control in certain parts of the world where copper salts are expensive or difficult to transport.

Mozley (1944) experimented with locally mined malachite applied either in bags of mosquito-netting, organdie or cheese-cloth, or by means of hand-pumps or motor sprayers, or as floating islands of malachite, sawdust and ground *Swartzia* pods. Malachite gave a 100 per cent. kill of *Biomphalaria* at concentrations of 1 : 2,500 in 18 hours, 1 : 5,000 in 24 hours, and 1 : 20,000 in 48 hours. From field experiments he concluded that malachite is effective in destroying *Physopsis* and *Biomphalaria* under the conditions found in Southern Rhodesia.

In an attempt to determine the molluscicidal action of various rocks containing copper, specimens were obtained from the Imperial Institute, London, and, after grinding by a metallurgical firm, were tested against *P. corneus*.

The ores, etc., obtained and investigated were as follows :

- A. Chalcocite (Cu_2S) and carrollite (CO_2CuS_4) from Northern Rhodesia.
- B. Chrysocolla ($\text{CuOSiO}_2 \cdot 2\text{H}_2\text{O}$) and malachite ($\text{CuCO}_3\text{Cu(OH)}_2$) from Northern Rhodesia.
- C. Malachite from Uganda.
- D. Chalcopyrite (CuFeS_2) and bornite (Cu_5FeS_4).
- E. Malachite from the Belgian Congo.
- F. Cuprite (Cu_2O) and malachite from the Belgian Congo.

TABLE I

Showing the effects of copper salts on *P. corneus* ; five snails were exposed each time

Substance	Dilution	Period of exposure	Result
Water (control)		24 and 48 hours	5 A
Copper sulphate	1 p.p.m.	24 hours	5 NR and M, but after 24 hours in clean water all A
	1 "	47 "	5 R, but M after 24-47 hours
	5 "	24 "	4 D, 1 R but M
	10 "	24 "	4 D, 1 R
	100 "	24 "	5 D
Copper acetate	1 p.p.m.	24 hours	3 A, 2 R but remained sluggish
	1 "	48 "	5 D
	5 "	24 "	1 R, 4 D
Copper chloride	1 p.p.m.	24 hours	1 D, 4 A after transfer to water
	1 "	46 "	2 D, 3 R after transfer to water
	5 "	24 "	5 D
Copper carbonate	1 p.p.m.	24 hours	5 A
	2 "	24 "	5 A
	5 "	24 "	1 D, 1 A, 3 R but M

A.—active ; M.—motionless ; R.—react to touch ; NR.—no reaction to touch ; D.—dead.

G. Native copper with cuprite, malachite and chrysocolla from the Belgian Congo.

H. Nickel-copper ore containing pyrrhotite (Fe_{1-x}S), pentlandite ($(\text{Fe,Ni})_9\text{S}_8$) and chalcopyrite.

I. Simple commercial metallic copper mesh, as used for scouring.

Using samples of these ores, etc., preliminary and somewhat empirical experiments were carried out, the results of which are shown in Table II.

From these experiments it appeared that the ores with the greatest toxicity were those containing malachite (basic or mineralized copper carbonate), with or without cuprite (Cu_2O), and chrysocolla ($\text{CuOSiO}_2 \cdot 2\text{H}_2\text{O}$).

The nickel-copper ore (H) containing pentlandite and pyrrhotite also killed, though its action was a little slower than that of the malachite ore. At high dilutions it had no effect.

It was concluded that certain copper ores have some degree of toxicity to snails. The toxicity is greatest in the case of malachite, less in the case of pentlandite and pyrrhotite (nickel-copper), and negligible in the case of chalcocite and chalcopyrite plus

TABLE II
Showing the effect of copper ores and commercial copper on *P. corneus*

Samples of copper used	No. of <i>P. corneus</i> in test	Form in which copper was used in aquarium	Results
Control	5	—	5 A
A	5	5 gm. ground*	5 A after 48 hours
B	5	5 gm. ground*	5 NR after 20 hours. On transference to clean water 2 D, 3 R but remained sluggish
C	5	5 gm. ground*	5 NR after 20 hours. On transference to clean water 4 D, 1 R but remained sluggish
C	10	5 gm. ground* in 4 litres of water	10 R at 24 hours; at 48 hours 2 R. On transference to clean water after 2 days 1 D, 7 R but M, 2 A
D	5	5 gm. ground*	5 A after 48 hours
E	5	5 gm. ground*	5 R at 20 hours. On transference to clean water 1 R, 4 D
E	10	5 gm. ground* in 4 litres of water	10 M at 24 hours, but R; at 48 hours only 8 R. On transference to clean water after 2 days 6 D, 4 R
F	10	Unmeasured quantity ground*	10 NR at 24 hours. On transference to clean water 10 A. On being replaced in test-aquarium for 2 days 10 M. After 2 days in clean water 6 D. Action slight
G	5	121 gm. solid ore in 250 c.cm. of water	5 NR after 20 hours. On transference to clean water at 24 hours 5 D
G	10	121 gm. solid ore in 4 litres of water	10 NR at 22 hours. On transference to clean water 7 hours later 2 R; next day 9 D, 1 R
H	5	5 gm. ground*	3 D at 48 hours, 2 R but subsequently D
H	10	5 gm. ground* in 4 litres of water	10 A after 2 days
I	10	33.4 gm. in 4 litres of water	10 NR after 5 hours. On transference to clean water at 24 hours 6 D, 4 R but subsequently D
I	10	5 gm. in 2 litres of water	10 NR after 24 hours. On transference to clean water at 50 hours 9 D, 1 A

A.—active; M.—motionless; R.—react to touch; NR.—no reaction to touch; D.—dead.

* Ores which had been ground were placed in parachute-silk bags which were gently moved up and down in the aquarium, containing 250 c.cm. of water, and then suspended in it.

bornite. Powdered malachite ores were toxic to snails at a 'dilution'—or, more correctly, suspension—of 5 gm. of ore in 4 litres of water, or 1.25 gm. per litre. Although the toxic value of ores is much lower than that of prepared soluble copper salts, they constitute one practicable possibility for snail control and are worthy of investigation in the field.

II. EXPERIMENTS WITH SUBSTANCES EXTRACTED FROM PLANTS

Molluscicidal principles have been extracted from a number of tropical plants.

SAPONINS

It is known that saponins, which are present in various plants, have some insecticidal value, and they are used by natives as fish poisons. Floch and Lajudie (1946) found that, in French Guiana, 50 *Aplexa marmorata* and 50 *Tropicorbis kuennianus* were killed in one and a half hours when placed in a litre of water containing 0.1 gm. of fruit pulp of *Sapindus saponaria* (= *S. arborescens*), which contains 66 per cent. of saponin.

In the present series of experiments saponin (Merck) was the first substance tested; the results are shown in Table III.

TABLE III

Showing the effect of varying dilutions of saponin (Merck) on *P. corneus*; five snails were used in each test

Concentration of saponin, in parts per million	Period of exposure of snails, in hours	Results
100	24	5 NR, M. On transference to clean water 5 R, M
10	24	3 D, 2 R
1	24	5 A
100	48	5 D
10	48	5 A
5	48	5 A
1	48	5 A

A.—active; M.—motionless; R.—react to touch; NR.—no reaction to touch; D.—dead.

As is shown in Table III, saponin in high dilutions has only a slight toxicity to snails and gave variable reactions. One hundred p.p.m. renders the snails prostrate and apparently moribund in 24 hours, and kills them effectively in 48 hours.

OTHER PLANT SUBSTANCES

Mozley (1944) investigated the action on snails of the following plants in Southern Rhodesia: *Swartzia madagascariensis* (a tree of the family Leguminosae), *Tephrosia vogelii*, *Eucalyptus* sp. and the Rhodesian tree *Capaifera mopane*.

It is well known that the various species of the African tree *Balanites* contain substances toxic to both fish and snails. In a recent report Ransford (1948), working in Nyasaland, has emphasized the value of *Tephrosia vogelii*, reporting that a 1 : 4,000 concentration of *Tephrosia* leaves and a 1 : 3,000 solution of *Swartzia madagascariensis* gave a 100 per cent. kill in 48 hours (*Physopsis globosa*). Mozley found that extracts of the pods and leaves of *S. madagascariensis* had a toxic effect on snails, but not in very high dilutions. His experiments with *Tephrosia vogelii* showed that leaves, stems and flowers all contained a principle lethal to *Physopsis*. He also found that extracts of the leaves, bark and sawdust of *Eucalyptus* sp. had some lethal effect on *Biomphalaria* (= *Planorbis*) and *Physopsis*, but only in strong concentrations. *Eucalyptus* sawdust had the same effect on *Physopsis*. Mopani leaves (leaves of *Capaifera mopane*) also contained a substance which killed *Physopsis*.

Through the courtesy of the curator of the Royal Botanic Gardens at Kew, specimens of some of these plants were obtained, extracts were made, and experiments with them

were conducted on *P. corneus*. *Swartzia madagascariensis* and *Copaifera mopane* were not obtainable, but experiments were carried out with the following: *Sapindus saponaria*, *Quillaja saponaria*, *Tephrosia vogelii*, *Eucalyptus* sp., *Balanites roxburghii* and *Randia vestita*. A few details, taken mainly from Dalziel (1937), Strasburger (1921) and Willis (1925), are given here regarding these plants.

Balanites roxburghii, family Simarubaceae. Occurs in India. Dalziel (1937) lists *B. aegyptiaca* and *B. wilsoniana* as growing in tropical Africa, where *B. aegyptiaca* is used by the natives as a fish poison. The roots have been used for snail-killing purposes in parts of Africa. The bark and roots probably contain a saponin.

Quillaja saponaria, family Rosaceae, the soap-tree of Chile. Contains a saponin, and the powdered bark lathers with water. An evergreen tree, with short-stalked, alternate, leathery leaves.

Sapindus saponaria, family Sapindaceae, the American soap-bush. The leaves and berries contain saponin.

Eucalyptus spp., family Papilionaceae. Much cultivated throughout tropical Africa as a fish poison. Both leaves and pods contain a toxic principle. Extracts of the leaves

TABLE IV

Showing the effect on *P. corneus* of the extracts of various plants; five snails were used in each experiment

Plant	Concentration of 4% extract in water	Hours of exposure	Results
<i>Eucalyptus</i> sp.	1 : 225	24	5 A
	1 : 5,000	46	2 A, 3 R but sluggish
<i>Quillaja saponaria</i>	1 : 250	24	5 D
	1 : 825	48	5 A
	1 : 5,000	46	4 A, 1 M
<i>Sapindus saponaria</i>	1 : 250	24	5 A
	1 : 5,000	46	5 A

A.—active; M.—motionless; R.—react to touch; D.—dead.

are also of value as a contact insecticide and have already been recommended as a molluscicide.

Randia vestita, family Rubiaceae. Dalziel (1937) records 11 species from tropical West Africa, where it has been used to provide both a fish poison and a therapeutic drug, e.g., for eye troubles. Willis (1925) states that 125 species of the genus are known, all of them tropical. Experiments in Tanganyika Territory have shown that the root-bark contains a saponin, and extracts have been used effectively in the treatment of gonorrhoea. Such extracts are said to contain a diuretic principle, and their use in blackwater-fever anuria has been suggested.

A preliminary experiment was carried out to compare the action of extracts of the various plants. Solutions were made up as follows:

Eucalyptus: 8 gm. of leaves were boiled in water, allowed to stand, and then filtered; the filtrate was made up to 200 c.cm. with water (4 per cent.).

Sapindus: 4 gm. of leaves were boiled in water, allowed to stand, and then filtered; the filtrate was made up to 100 c.cm. with water (4 per cent.).

Tephrosia: 5 gm. of leaves were boiled, allowed to stand, and then filtered; the filtrate was made up to 500 c.cm. with water (1 per cent.).

Quillaja saponaria: 4 gm. of leaves and petioles were boiled, allowed to stand, and then filtered; the filtrate was made up to 100 c.cm. with water (4 per cent.).

Balanites roxburghii: 15 gm. of the pericarp of the fruit were boiled, filtered, and made up to 300 c.cm. with water (5 per cent.); 2 gm. of kernel (seed) were also boiled, filtered, and made up to 200 c.cm. with water (1 per cent.).

The effect of these extracts of the various plants on snails was investigated first for purposes of comparison, after which the different plants were tested individually. Experiments are described below in series.

Quillaja saponaria

It was noted that, as is shown in Table IV, a 0.4 per cent. solution of *Q. saponaria* appeared to kill *P. corneus* in 24 hours, and accordingly this plant was investigated first.

TABLE V

Showing the effect of various concentrations of extract of the leaves of *Quillaja saponaria* on *P. corneus*; five *P. corneus* were exposed in each test, and in the 48-hour tests one *A. glabratus* was exposed with each batch of *P. corneus*

Concentration of extract in water	Hours of exposure	Results
1 : 2,500	24	5 A
1 : 500	24	4 D, 1 A
1 : 250	24	5 D
1 : 167	24	5 D
1 : 125	24	5 D
1 : 10,000	48	6 A
1 : 5,000	48	6 NR. On transference to clean water 6A
1 : 2,000	48	6 D

A.—active; NR.—no reaction to touch; D.—dead.

There seems to be a toxic principle in *Quillaja* leaves and petioles which kills in 24 hours at 0.2 per cent. (2,000 p.p.m.) upwards, or in 48 hours at dilutions of 0.05 per cent. (500 p.p.m.).

The results of experiments with broken and unbroken leaves, bark and stripped wood of *Q. saponaria* on *P. corneus* are summarized in Table VI. Leaves are more toxic when crushed or broken. The stripped bark appeared to have the greatest toxic effect.

To test the toxic action of stripped green wood and of the bark of *Q. saponaria* on *P. corneus*, 10 gm. of stripped green wood were boiled in water, and the solution was filtered and made up to 200 c.cm. From this the test-solutions were made up. And 5 gm. of freshly stripped bark were extracted with 100 c.cm. of water and boiled for half an hour. The solution was then filtered and made up to 200 c.cm. The results of this experiment are shown in Table VII.

From these experiments it appears that *Quillaja* bark contains a substance toxic to *P. corneus* at 0.05 per cent. and lethal at 0.62 per cent. The stripped wood contains a substance toxic at 1 per cent. and lethal at 2 per cent. The leaves contain a substance

TABLE VI

Showing the effect on *P. corneus* of broken and unbroken leaves, bark and stripped wood of *Quillaja saponaria*; five snails were exposed in each test

Quantity and nature of the toxic agent	Period of exposure, in hours	Results
Tap-water (control)	24 and 48	5 A
<i>Quillaja saponaria</i>		
1.5 gm. of fresh stripped bark in 200 c.cm. water	24	5 NR. After 24 hours in water 5 R; 2 days later 5 D
6 large fresh unbroken leaves in 200 c.cm. water	48	5 A (2 M but R)
5 gm. of green freshly stripped wood in 200 c.cm. water	24	5 M. After 24 hours in water 5 M, but 4 R; 18 hours later 2 D, 3 R
5 crushed leaves in 200 c.cm. water	48	5 M, but 4 R after 24 hours; after 48 hours 5 D
6 gm. of crushed green stems in 200 c.cm. water	70	4 A, 1 M after 24 hours; after 70 hours 5 M. On transference to clean water 5 A but 2 sluggish
5 gm. of stripped green wood, which had been dried for 24 hours, in 200 c.cm. water	22	5 M. After 48 hours in water 5 A
1.5 gm. of stripped bark, which had been dried over night, in 200 c.cm. water	70	5 M after 22 hours; after 70 hours 5 D

A.—active; M.—motionless; R.—react to touch; NR.—no reaction to touch; D.—dead.

both toxic and lethal at 0.2 per cent. in 24 hours or at 0.05 per cent. in 48 hours. The leaves and the bark are more toxic than the wood. Crushed or broken leaves are more effective than unbroken leaves. The toxicity of the bark was not impaired by drying over night, and the toxicity of the wood was impaired only slightly, if at all, by drying.

Balanites roxburghii

The next plant to be investigated was *B. roxburghii*. As no fresh *Balanites* were available, some fruits were used which had been preserved dry for some years—probably for as long as 10 years. Extracts were made of the dry pericarp and kernel (i.e., seed).

TABLE VII

Showing the effect on *P. corneus* of extracts of stripped green wood and bark of *Quillaja saponaria*; two snails were exposed in each test

Extract	Proportion of plant substance to final dilution of extract	Period of exposure, in hours	Results
Stripped green wood	1 : 40	24	2 D
	1 : 100	24	1 D, 1 A on transference to clean water
	1 : 200	48	2 A
	1 : 400	48	2 A (1 R but M)
	1 : 2,000	48	2 A
Bark	1 : 40	22	2 D
	1 : 80	22	2 D
	1 : 160	22	2 D
	1 : 2,000	22	2 R. After transference to clean water 1 A, 1 D
	1 : 4,000	22	2 A

A.—active; M.—motionless; R.—react to touch; D.—dead.

TABLE VIII

Showing the effect of various concentrations of the extract of dried pericarp of *Balanites roxburghii* on *P. corneus* and *A. glabratus*; five *P. corneus* and two *A. glabratus* were used in each test

Proportion of dried pericarp to final dilution of extract	Results
1 : 100	7 D
1 : 200	7 D
1 : 400	7 D
1 : 2,000	7 D
1 : 4,000	7 M. On transference to clean water 7 R
1 : 20,000	7 A
1 : 40,000	7 A
1 : 200,000	7 A
1 : 400,000	7 A
1 : 1,000,000	7 A

A.—active ; M.—motionless ; R.—react to touch ; D.—dead.

Fifteen gm. of dried pericarp were boiled in water, and the filtered extract was made up to 300 c.cm. with water. From this the test-solutions were prepared.

The stock solution of *Balanites* kernel was made up by extracting 2 gm. of the kernel in boiling water, filtering, and making up to 200 c.cm. with water. The dilutions were made from this stock solution.

From these experiments it appears that *B. roxburghii* contains a principle toxic to the snails and present both in the pericarp and in the kernel. It is not destroyed by drying for some years. The toxic action of pericarp extract was appreciably greater than that of the extract of kernel. Leaves, bark and roots were not available for experiment.

Eucalyptus spp.

The leaves of various species of *Eucalyptus* (gum-tree) were used. An extract of 3 gm. of leaf was, after a brief boiling, made up to 300 c.cm. with water, and the snails were exposed to solutions varying from 0.5 per cent. to 0.1 per cent., these solutions being dilutions of the above 3 per cent. solution. None of the snails died within 24 hours. Of five exposed to a 0.5 per cent. solution two were quiescent, though they remained attached to the glass, while three were apparently moribund, though they reacted to touch. In

TABLE IX

Showing the effect during a period of 48 hours of various concentrations of the extract of the kernel of *Balanites roxburghii* on *P. corneus* and *A. glabratus*; six or seven snails were used in each test

Proportion of kernel to final dilution of extract	Results
1 : 100	6 M. On transference to clean water for 48 hours 1 R, 5 D
1 : 200	7 D
1 : 1,000	3 D, 3 R
1 : 10,000	7 A but sluggish
1 : 100,000	7 A
1 : 200,000	7 A
1 : 1,000,000	7 A

A.—active ; M.—motionless ; R.—react to touch ; D.—dead.

a 0.2 per cent. solution two appeared moribund but just reacted to touch. In the remaining solutions most of the snails were still able to cling to the sides of the glass, though they were sluggish and quiescent. One or two appeared prostrate but reacted to touch. After 45 hours the snails were still alive, though two of those in the 0.2 per cent. solution appeared moribund.

All the snails used were *P. corneus*, and it was concluded that the toxic action of *Eucalyptus* extract on this snail is negligible at any practical dilution.

Sapindus saponaria

An extract of the leaves of *S. saponaria* was made by boiling and was tested against five *P. corneus* in each test, the concentrations varying from one part of leaves per 100 final dilution of the extract to one part per 4,000. Though the stronger concentrations proved toxic to the snails, none were lethal, and recovery invariably occurred on transference to clean water. It was concluded that the action of *S. saponaria* on *P. corneus* is negligible at any practical dilution.

Tephrosia (probably *T. vogelii*)

A consignment of *Tephrosia* leaves was received through the kindness of Dr. Ransford, of the Nyasaland Medical Service. A stock solution was made by boiling 5 gm. of the leaves in water, and, after filtration, by making the extract up to 500 c.cm. No ill effects were obtained among 10 *P. corneus* exposed to each of a series of concentrations of leaf to final dilution ranging from 1 : 10,000 to 1 : 500,000 over periods of 25–48 hours' exposure. Snails exposed to 10 lightly crushed leaves in 500 c.cm. of water were not killed, though they appeared sluggish after 48 hours' exposure. Ten snails exposed to 20 lightly crushed leaves in 500 c.cm. of water showed no ill effects after 24 hours and 48 hours. Two only remained alive after 72 hours' exposure. Similar experiments with *A. glabratus* and *B. tropicus* produced similar results. Increasing the concentration of the toxic principle by adding lightly crushed leaves to the stock solutions confirmed the impression that *Tephrosia* leaves are toxic to these snails only in very strong concentrations, and then only after prolonged exposure.

Randia vestita

The last plant-product tested was extract of the root-bark of *Randia vestita* from Tanganyika, a sample of which was kindly supplied by Dr. F. Hawking, of the National Institute for Medical Research, Hampstead, who received it from Dr. H. Fairbairn, of the Sleeping Sickness Department in Tanganyika.

Ten gm. of the bark were boiled for a short time in water and the filtered solution was made up to 200 c.cm. Test-dilutions were made from this 5 per cent. stock extract.

Five *P. corneus* were exposed to 200 c.cm. of solutions containing 2.5 per cent., 1 per cent., 0.5 per cent., 0.1 per cent., 0.05 per cent., 0.025 per cent. and 0.01 per cent. of extract made up as above. At 2.5 per cent., 1 per cent. and 0.5 per cent. all snails appeared dead after 24 hours, and there was no recovery after transference to clean water. All snails survived 48 hours' exposure to dilutions of 0.1–0.01 per cent. without ill effects.

It is concluded that the root-bark of *R. vestita* is lethal to *P. corneus* at dilutions down to 0.5 per cent. (5,000 p.p.m.).

SUMMARY

1. A review is made of part of the literature dealing with snail control, particularly through the use of copper.

2. Experiments with copper salts and crude copper ores were carried out on the English pond snail *Planorbis corneus*. Of the salts tested the sulphate, acetate and chloride were all effective against *P. corneus*. Metallic copper and certain copper ores, especially those containing malachite, were found to be lethal, or at least toxic, to *P. corneus*.

3. Preparations and extracts of certain tropical plants, including *Eucalyptus* spp., *Quillaja saponaria* (the Chilean soap-tree), *Sapindus saponaria* (the American soap-bush), *Balanites roxburghii*, and the root-bark of *Randia vestita*, were tested against *P. corneus*. The action of *Eucalyptus* and *Sapindus* was almost negligible; *Balanites*, *Randia* and *Quillaja* contain a molluscicidal principle, which was investigated in further detail; *Tephrosia* leaf was effective only in high concentrations over long periods.

4. The effect of saponin was also investigated and was found to be slight.

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EXPERIMENTS IN CROSS-BREEDING TSETSE-FLIES (*GLOSSINA* SPECIES)

BY

F. L. VANDERPLANK

(Research Officer (Entomologist), Tsetse Research Organization, East Africa)

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INTRODUCTION

Stuhlmann (1907) claimed to have observed parthenogenesis on two occasions in *Glossina brevipalpis* (Newstead), a claim which has not been confirmed with this or any other species. Kleine (1909) crossed *G. palpalis* (*fuscipes*) (Newstead) with '*G. morsitans* (Westwood)' (his '*G. morsitans*' was most probably *G. swynnertoni* (Austen), since the pupae were collected in the *swynnertoni* bush of Ikoma, Tanganyika Territory), but he failed to obtain any offspring. Corson (1932) obtained hybrids by crossing *G. morsitans morsitans* (Vanderplank) with *G. swynnertoni*. Other published results on this particular cross in the laboratory and in the field have been given by Potts (1937, 1940, 1944), Jackson (1945, 1946) and Vanderplank (1944, 1945, 1947a, 1947b).

The following account of experiments on the cross-breeding and attempted cross-breeding of various species and subspecies of tsetse-flies is taken from a dissertation (which includes maps giving the distribution of the various species) for the degree of Ph.D. submitted by the writer to the University of Bristol in October, 1947. The distribution and a description of the various subspecies and hybrids of *morsitans* are also given by Vanderplank (1948a), for *G. longipalpis* (Wiedmann), *G. pallidipes* (Austen) and *G. austeni* (Newstead) by Vanderplank (1948b), and for the subspecies and hybrids of *G. palpalis* by Vanderplank (1948c).

The experiments were carried out from 1943 to 1947 at Shinyanga, Tanganyika Territory, and at the Department of Zoology in the University of Bristol. Some of the previously published results on the cross-breeding of *morsitans* with *swynnertoni* are, for comparative purposes, repeated in the tables of the present paper. Other observations on cross-breeding *pallidipes* collected in different localities, and on *G. palpalis palpalis* and *G. palpalis martinii* from two localities, have not been repeated, as they are included in Vanderplank (1948b) and Vanderplank (1948c) respectively.

Material has been collected as pupae from the following localities: *G. morsitans morsitans* at Kondoia Irangi, Tanganyika, and Ankole, Uganda; *G. morsitans orientalis* (Vanderplank) from Kingolwira, Morogoro, Tanganyika; *G. swynnertoni* from Shinyanga, Tanganyika; *G. pallidipes* from Kisii, Kenya, and from Shinyanga and Kingolwira, Tanganyika; *G. austeni* from Kingolwira; *G. morsitans submorsitans* (Newstead) from Kaduna, Nigeria; *G. brevipalpis* from Kisii, Kenya, and Kingolwira; *G. fuscipleuris* (Austen) from Kisii, Kenya; *G. longipennis* (Corti) from Mbulu, Tanganyika; *G. palpalis martinii* (Zumpt) from Mpulungu, Abercorn, Northern Rhodesia, and from Kasanga, Tanganyika; *G. palpalis fuscipes* from Entebbe, Uganda; *G. palpalis palpalis* (Rob.-Des.) and *G. tachinoides* (Westwood) from Kaduna, Nigeria, and from Tamale, Gold Coast.

INTERSPECIFIC AND INTERGROUP MATINGS

GENERAL CONSIDERATIONS

Twelve species and over 3,000 different individuals have been individually mated, maintained and observed during the course of five years' experimental work. Many specimens failed to copulate for more than a few minutes, and these were generally killed and dissected; the majority were found not to be inseminated, though a few sperms were found in occasional females. Including the hybrids F_1 and F_2 , some 108 different types of crossings were made, involving a total of just over 1,300 females, or an average of approximately 12 individuals for each type of cross. In actual fact many crosses recorded rest on only one female, which is quite useless for any estimation of results, beyond establishing whether or not two species are able to mate and whether the female can be inseminated successfully. A positive result, especially with the production of offspring, is of value, but a negative result proves little beyond the fact that the two species have been observed in coitus. Samples of at least 100, and preferably 500, are desirable before any definite conclusions can be deduced; but so far it has been possible in only one type of cross to obtain a sample of as many as 100. The difficulties of keeping a large number of tsetse in single tubes have been pointed out elsewhere (Vanderplank, 1947a). Even when difficulties of feeding were overcome it was found that only 300 inseminated females could be handled at any one time, owing to the large amount of work involved daily in recording the data. A record has been kept of every individual fly, whether fed or unfed, of air-temperatures and humidities, and of other relevant data. The average life of each female is about 10-12 weeks, which would make it theoretically possible to handle some 1,200 flies a year; but there are seasonal difficulties in obtaining pupae, other than *G. m. morsitans* from Kondoa Irangi, Tanganyika, and *G. p. fuscipes* from Uganda, as other species can only be collected for short periods, generally at the end of the dry season. Nevertheless, in spite of the shortcomings in the numbers of crosses made, a mass of useful data has been collected. This sets something of a problem in presentation, since, if each cross were dealt with separately, a great deal of repetition would be unavoidable. All the data, therefore, have been summarized in tabular form and are given in Table I. These data as a whole are discussed first, followed by consideration of interspecific differences, intergroup differences, and other points of interest. The methods used are given by Vanderplank (1947b).

I. PRODUCTIVITY OF CONTROLS

Throughout these experiments, numerous intraspecific (control) crosses were carried out to give an indication of the productivity of each species under laboratory conditions. For example, as shown by the tables, intraspecific crosses with *G. p. fuscipes*, *G. p. martinii*, *G. m. morsitans* from Ankole, *G. m. submorsitans* and *G. austeni* showed a 100 per cent. productivity. This percentage of productivity is based on the numbers which were proved by dissection at death to have been inseminated and which survived for at least 21 days, the time required for production of the first larva. Although these species all produced at least one offspring apiece, they did not average their full possible number of offspring: for example, in *G. p. fuscipes* the average number of pupae per female was 2.3, but each of these females lived on an average 66 days, and, had they produced their maximum number of offspring, their average number of pupae would have been 5.5. Moreover, not all the intraspecific crosses showed a productivity of 100 per cent.: in *G. p. palpalis*,

for instance, only 22 of the 24 inseminated females that lived for 21 days or more actually produced any pupae; and, though their average of 5.5 per female is the highest recorded for any species, nevertheless, since the average life of these females was 84 days, the maximum or expected number would be in the neighbourhood of 7.3 pupae per female. *G. m. orientalis* was the least productive of all the species kept to date, only 25 per cent. of the females producing any offspring at all, and those which did producing only one pupa per female. The productivity of these control females must be taken into account in the evaluation of results of interspecific and intergroup crosses.

II. PRODUCTIVITY OF INTERSPECIFIC CROSSES

In interspecific crosses at least five different considerations must be taken into account when estimating the productivity of the females.

1. Failure to Copulate

Unless the mating-notes (wing-beat, flying or stationary) of the two species to be crossed overlap in their frequency range, no attempt to mate will, under normal conditions, be made by the two species. Such mating can, however, be brought about by two methods.

(a) If a number of males of species A and species B are mixed in a cage (similar to that described by Vanderplank, 1947a), and if a mixed number of gorged females of the same two species are suddenly introduced, most of the pairing which takes place will be intraspecific, but a percentage—varying with the experiment, but averaging about 5 per cent.—will be interspecific, male A mating with female B and male B mating with female A, cross-mating apparently being brought about in the general confusion of mass pairing.

(b) Interspecific and intergroup matings can be effected by sounding the frequency of the note which is usually made by a virgin female of the same species as the male when the male is introduced into a tube to a strange virgin. It is necessary to sound this note, which can be produced either mechanically or from an actual record of a virgin fly, continuously or intermittently throughout the time required for normal coitus.

Previous workers have experienced considerable difficulty in mating species of *palpalis* in captivity. Investigation has shown that this is due either to unattractiveness or to resistance on the part of the female. However, as I have described elsewhere (Vanderplank, 1947b), females are unable to resist coitus (a) during the first hour or two after emergence, and (b) immediately after their first or subsequent meal. Females usually resist by bending their abdomens away from the male, but at these times they are unable to do so. A virgin becomes unattractive to males after she is 10–20 days old, owing to the change in wing-beat frequency, and a female becomes unattractive to males 24 hours after successful insemination. Moreover, on account of the change in the vagina of inseminated females, true coitus becomes impossible after insemination has taken place. The range of wing-beat frequencies of some species, such as *G. swynnertoni*, and of subspecies of *morsitans* and *palpalis*, overlap, and mating readily takes place where the overlap is considerable, as well as, to a lesser degree, in other cases. Hence *G. swynnertoni* and *G. m. morsitans* mate almost at random, and *G. swynnertoni* and *G. m. orientalis* mate together more readily than with their own species; *G. m. orientalis* and *G. m. morsitans*, on the other hand, mate together less readily, *G. swynnertoni* and *G. m. submorsitans* are very reluctant to mate and have to be encouraged to do so, and *G. m. submorsitans* and *G. m. morsitans* mate at random when the males are *submorsitans* but are reluctant when the males are *morsitans*.

TABLE I
Summary of intra- and interspecific crosses of *Glossina*

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Reference no. of species	Species mated	No. of pairs mated	No. proved inseminated	Percentage proved inseminated of those mated	No. inseminated and living 21 days or more	Percentage of those inseminated and living 21 days or more over no. inseminated	No. of females producing pupae	As percentage of those inseminated and living 21 days or more	Total no. of pupae produced	Mean per inseminated female that lived 21 days or more	Maximum longevity of females	Mean longevity of females	Remarks
PALPALIS GROUP													
CONTROLS													
PaPa	<i>G. p. palpalis</i> × <i>G. p. palpalis</i>	44	32	73%	24	75%	22	92%	130	5.5	154	84	Ex Nigerian pupae
FF	<i>G. p. fuscipes</i> × <i>G. p. fuscipes</i>	41	30	73%	22	71%	22	100%	51	2.3	143	66	Ex Uganda pupae
TT	<i>G. p. martinii</i> × <i>G. p. martinii</i>	22	18	82%	17	94½%	17	100%	51	3.0	106	60	Ex N. Rhodesian pupae
TaTa	<i>G. tachinoides</i> × <i>G. tachinoides</i>	9	6	67%	6	100%	5	33%	27	4.5	119	70	Ex Nigerian pupae
CROSSES													
F Pa	<i>G. p. fuscipes</i> × <i>G. p. palpalis</i>	29	28	97%	24	86%	6	25%	12	0.5	79	41	Intergroup cross
T Pa	<i>G. p. martinii</i> × <i>G. p. palpalis</i>	38	36	95%	7	19%	3	43%	5	0.7	73	50	
D Pa	<i>G. pallidipes</i> × <i>G. p. palpalis</i>	1	1	(100%)	1	(100%)	0	—	0	—	32	(32)	
MaPa	<i>G. m. morsitans</i> × <i>G. p. palpalis</i> (Ankole)	1	1	(100%)	1	(100%)	0	—	0	—	31	(31)	
TaPa	<i>G. tachinoides</i> × <i>G. p. palpalis</i>	1	1	(100%)	1	(100%)	0	—	0	—	42*	(42)	
PaF	<i>G. p. palpalis</i> × <i>G. p. fuscipes</i>	64	61	95%	45	75%	2	4.4%	3	0.07	64	47	After male or female had been treated to prevent injury by male claspers
T F	<i>G. p. martinii</i> × <i>G. p. fuscipes</i>	145	100	69%	20	20%	10	50%	17	0.35	144	61	Intergroup cross
I F	<i>G. m. morsitans</i> × <i>G. p. fuscipes</i> (Kondoa)	4	4	100%	4	100%	0	—	0	—	32*	29	Intergroup cross
X F	<i>G. submorsitans</i> × <i>G. p. fuscipes</i>	12	12	(100%)	12	(100%)	0	—	0	—	82	52	" "
S F	<i>G. swynnertoni</i> × <i>G. p. fuscipes</i>	6	6	(100%)	6	(100%)	0	—	0	—	101	58	" "
PaT	<i>G. p. palpalis</i> × <i>G. p. martinii</i>	22	19	86%	18	95%	4	22%	10	0.56	85	47	" "
F T	<i>G. p. fuscipes</i> × <i>G. p. martinii</i>	25	24	96%	23	96%	5	22%	5	0.22	154	59	" "

* Killed

TABLE I (Continued)

1	2	3	4	5	6	7	8	9	10	11	12	13	14
I T	<i>G. m. morsitans</i> × <i>G. p. martinii</i> (Kondoa)	4	4	100%	4	100%	0	—	0	—	66	39	Inter group cross " "
X T	<i>G. submorsitans</i> × <i>G. p. martinii</i>	1	1	(100%)	1	(100%)	0	—	0	—	31	(31)	
Pa Ta	<i>G. p. palpalis</i> × <i>G. tachinoides</i>	1	1	(100%)	1	(100%)	0	—	0	—	51	(51)	
Wild, W F	collected in the field <i>G. p. fuscipes</i> caught at Ukerewe Isle	25	24	96%	13	54%	11	85%	18	1.6	81	45	
Virgin, G P	released in thorn bush in Shinyanga <i>G. p. fuscipes</i> ex Uganda pupae	12	10	83%	7	70%	5	71%	8	1.6	81	57	
PALPALIS-GROUP HYBRIDS													
(i) Hybrids × Hybrids													
O O	<i>G. 'martipes'</i> × <i>G. 'martipes'</i> ex T F cross	2	2	100%	2	100%	2	100%	5	2.5	58	75	After cutting claspers " "
R R	<i>G. 'fuscitini'</i> × <i>G. 'fuscitini'</i>	3	2	67%	2	100%	1	50%	1	0.5	92	67	
—	<i>G. 'paltinii'</i> × <i>G. 'paltinii'</i>	2	22	100%	2	100%	0	—	0	—	98	70	
(ii) Hybrids × Pure-Breds													
Some 50 crosses have been carried out, using male hybrids, <i>G. 'martipes'</i> (ex T F cross), <i>G. 'fuscitini'</i> (ex F T cross), <i>G. 'paltinii'</i> (ex Pa T cross), <i>G. 'marpatis'</i> (ex T Pa cross), <i>G. 'fuspalis'</i> (ex F Pa cross), and <i>G. 'paltipes'</i> (ex Pa F cross), crossed with various pure-bred females, and using pure-bred males crossed with hybrid females. These crosses showed that hybrids are at least partly, if not entirely, fertile, and second generation offspring, which also proved fertile, was obtained.													
MORSITANS GROUP													
CONTROLS													
I I	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> ex Kondoa	69	56	81%	42	52%	40	95%	112	2.7	113	72	
Ma Ma	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> ex Ankole	3	3	100%	3	100%	3	100%	3	1.0	49	34	
K K	<i>G. m. orientalis</i> × <i>G. m. orientalis</i> ex Kingolwira	6	4	67%	4	100%	1	25%	4	1.0	110	74½	
X X	<i>G. m. submorsitans</i> × <i>G. m. sub-</i> ex Nigeria <i>morsitans</i>	6	6	100%	6	100%	6	100%	11	1.8	56	41½	
S S	<i>G. scymnertoni</i> × <i>G. scymnertoni</i> ex Shinyanga	60	48	80%	27	80%	16	59%	29	1.1	114	58	
D D	<i>G. pallidipes</i> × <i>G. pallidipes</i> ex Shinyanga	5	3	60%	3	100%	1	33%	3	1.0	106	53	
A A	<i>G. austeni</i> × <i>G. austeni</i> ex Kingolwira	8	8	100%	6	75%	6	100%	25	4.1	136	66	
Wild females collected in the field													
KWA	<i>G. austeni</i>	34	34	100%	25	74%	24	96%	84	3.4	131	58	
KWK	<i>G. m. orientalis</i>	9	9	100%	9	100%	6	67%	11	1.8	97	58	

TABLE I (Continued)

I	2	3	4	5	6	7	8	9	10	11	12	13	14
KWD	<i>G. pallidipes</i> ex Kingolwira	6	6	100%	5	83%	4	80%	10	2.5	69	45½	
WD	<i>G. pallidipes</i> ex Shinyanga	4	3	75%	2	67%	2	100%	6	3.0	64	49	
WS	<i>G. swynnertoni</i> ex Shinyanga	10	10	100%	7	70%	6	86%	21	3.5	86	64½	
Females	released as virgins in thorn bush (Shinyanga), then recaptured and kept in the laboratory	29	27	93%	24	89%	14	38%	36	1.5	111	67	
FM	<i>G. m. morsitans</i> ex Kondeo pupae												
CROSSES													
SI	<i>G. swynnertoni</i> × <i>G. m. morsitans</i> ex Kondeo	91	82	91%	59	72%	14	24%	21	0.36	122	81½	Hybrids sterile
KI	<i>G. m. orientalis</i> × "	70	66	94%	54	82%	16	30%	20	0.37	113	59	" (E)
XI	<i>G. submorsitans</i> × "	31	24	79%	14	58%	3	21%	3	0.21	65	41	"
MaI	<i>G. m. morsitans</i> × "	8	4	50%	4	100%	3	75%	5	1.25	48	28½	Offspring fertile
FI	<i>G. p. fuscipes</i> × "	4	4	100%	4	100%	0	—	0	—	330	29	Inter-group cross
TI	<i>G. p. martinii</i> × "	9	9	100%	2	22%	0	—	0	—	69	49	"
AI	<i>G. austeni</i> × "	3	2	67%	1	(50%)	0	—	0	—	65	51	One with larva in uterus
DI	<i>G. pallidipes</i> × "	44	3	75%	3	100%	0	—	0	—	59	39	Offspring fertile
I Ma	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> (Ankole)	4	4	100%	4	100%	4	100%	8	2.0	69	41	Hybrids sterile (C)
X Ma	<i>G. m. submorsitans</i> × "	5	4	80%	4	100%	0	—	0	—	55	38	"
I K	<i>G. m. morsitans</i> × <i>G. m. orientalis</i> (Kondeo)	10	10	100%	9	90%	6	67%	18	2.0	121	69	"
SK	<i>G. swynnertoni</i> × "	23	23	100%	19	83%	11	58%	25	1.3	150	105	Hybrids sterile (G)
MaX	<i>G. m. morsitans</i> × <i>G. submorsitans</i> (Ankole)	25	24	96%	24	100%	12	50%	17	1.4	100	73	"
I X	<i>G. m. morsitans</i> × "	24	24	100%	18	75%	6	33%	6	1.0	96	68	Hybrids sterile
I S	" × <i>G. swynnertoni</i>	100	92	92%	80	87%	5	6.2%	5	0.06	112	69	"
KS	<i>G. m. orientalis</i> × "	54	50	92%	35	70%	3	8.6	5	0.14	142	75	"
AS	<i>G. austeni</i> × "	4	3	75%	0	—	0	—	0	—	0	21	"
KA	<i>G. m. orientalis</i> × <i>G. austeni</i>	1	1	(100%)	1	(100%)	0	—	0	—	109	(109)	"
SX	<i>G. swynnertoni</i> × <i>G. m. submorsitans</i>	2	2	(100%)	2	100%	0	—	0	—	73	54	First stage larva not viable
X S	<i>G. submorsitans</i> × <i>G. swynnertoni</i>	12	12	100%	12	100%	0	—	0	—	85	62	"
MORSITANS-GROUP HYBRIDS F ₁													
(i) Hybrids × Hybrids													
CC	<i>G. morsitans</i> × <i>G. morsitans</i>	2	2	100%	2	100%	0	0%	0	0	119	99	
EE	KI hybrid × KI hybrid	1	0	0%	0	0%	0	0%	0	0	6	—	
GH	SK cross × KS cross	1	0	0%	0	0%	0	0%	0	0	39	—	

TABLE I (Continued)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	
(ii) Pure-Breds × Hybrids														
IC	<i>G. m. morsitans</i> × <i>G. morsityhybrid</i>	2	2	100%	2	100%	1	50%	3	1.5	97	92.5	Male hybrids apparently sterile	
IR	" × KI cross	1	0	0%	0	0%	0	0%	0	0	17	—		
IN	" × IS cross	1	1	100%	1	100%	0	0%	0	0	32	32		
KC	<i>G. m. orientalis</i> × <i>G. morsityhybrid</i> (C)	11	1	100%	1	100%	0	0%	0	0	80	80		
KG	" × KS hybrid	2	1	50%	1	100%	0	0%	0	0	115	115		
SE	<i>G. swynnertoni</i> × <i>G. morsityhybrid</i> (E)	1	1	100%	1	100%	0	0%	0	0	69	69		
SG	" × KS hybrid	2	22	100%	2	100%	1	50%	5	2.5	152	93.5		
(iii) Hybrids × Pure-Breds														
CI	GK cross × <i>G. m. morsitans</i>	17	14	82.4%	7	50%	0	0%	0	0	95	41		
CK	IK cross × <i>G. m. orientalis</i>	1	1	100%	1	100%	0	0%	0	0	79	79		
CS	<i>G. morsityhybrid</i> × <i>G. swynnertoni</i>	4	4	100%	2	50%	0	0%	0	0	121	79		
EI	KI cross × <i>G. m. morsitans</i>	10	6	60%	3	50%	0	0%	0	0	78	35		
EK	" × <i>G. m. orientalis</i>	2	2	100%	2	100%	0	0%	0	0	81	80.5		
ES	KI hybrid × <i>G. swynnertoni</i>	12	12	100%	8	67%	0	—	0	0	50	37		
GA	SK cross × <i>G. austeni</i>	2	2	100%	2	100%	0	—	0	0	87	74		
GI	SE cross × <i>G. morsitans</i>	5	4	80%	4	100%	0	—	0	0	61	53		
GK	SK hybrid × <i>G. m. orientalis</i>	4	4	100%	4	100%	0	—	0	0	130	95½		
GS	SK cross × <i>G. swynnertoni</i>	8	7	87.5%	7	100%	0	—	0	0	112	51		
JI	SI cross × <i>G. morsitans</i>	4	4	100%	4	100%	0	—	0	0	31	54		
	IX and XI cross × IX and XI cross	3	3	100%	3	100%	0	—	0	0	89	48		
	IX and XI cross × <i>G. m. morsitans</i> (Kondoa)	15	15	100%	10	67%	0	—	0	0	125	72		
	IX and XI cross × <i>G. m. submorsitans</i> (Kaduna)	12	12	100%	12	100%	0	—	0	0	145	84		
MORSITANS-GROUP HYBRIDS F ₂														
SV	<i>G. swynnertoni</i> × SG hybrid	1	1	100%	0	0%	0	0%	0	0	8	—	Ex Kisii, Kenya, pupae Ex Mbulu pupae Not inseminated	
KV	<i>G. m. orientalis</i> × SG hybrid	2	2	100%	0	0%	0	0%	0	0	151	92		
IV	<i>G. morsitans</i> × SG hybrid	1	0	0%	0	0%	0	0%	0	0	37	—		
KX	<i>G. m. orientalis</i> × IC hybrid	1	1	100%	0	0%	0	0%	0	0	4	—		
CONTROLS														
Δ Δ	<i>G. fuscipileuris</i> × <i>G. fuscipileuris</i>	5	3	60%	3	100%	1	33%	1	0.33	71	42		
β β	<i>G. brevipalpis</i> × <i>G. brevipalpis</i>	9	9	100%	4	44%	2	50%	5	2.5	100	56.4		
φ φ	<i>G. longipennis</i> × <i>G. longipennis</i>	11	11	100%	10	91%	8	80%	15	1.5	107	64.8		
CROSSES														
Δ β	<i>G. fuscipileuris</i> × <i>G. brevipalpis</i>	1	0	0%	—	—	—	—	—	—	51	}		
β Δ	<i>G. brevipalpis</i> × <i>G. fuscipileuris</i>	1	0	0%	—	—	—	—	—	—	40			

Similarly, in the subspecies of *G. palpalis*, *G. p. martinii* prefers *G. p. fuscipes* to its own species and mates at random with *G. p. palpalis*, while *G. p. fuscipes* mates readily, though not at random, with *G. p. palpalis* but is reluctant to mate with *G. p. martinii*; male *G. p. palpalis* appear to mate at random with both subspecies. The only other species which will intermate at random, although they are seldom able to copulate successfully, are *G. tachinoides* and *G. pallidipes*. None of these species or subspecies, except *swynnertoni* and *morsitans*, meet in the field.

As already pointed out, the mating barrier can be artificially overcome in the laboratory; but there are several other barriers to successful insemination and production of offspring.

2. Damage During Copulation

Although three subspecies of *palpalis* will intermate with each other, the result of some of the combinations terminates with the death of the female, owing to the superior

TABLE II

Showing the numbers and percentages of females killed in interspecific matings with male *martinii* and male *palpalis*

Cross	No. mated, omitting treated females and escapes	No. killed by males	No. not inseminated	No. inseminated and living 21 days or more	Percentage surviving and capable of reproduction
TF series	118	105	13	0	0
TI "	8	7	1	0	0
TPa "	38	31	1	6	15.8
PaF "	64	17	3	44	69
PaT series	22	1	3	18	82

claspers of the male piercing the abdomen and gut, and occasionally even protruding on the dorsal side of the female—as invariably happens when male *martinii* mate with female *fuscipes*. Moreover, in females which have mated for a short time and have not been injured by the male claspers, it is also found that the male has failed to inseminate them. Table II shows the numbers and percentages of females killed in these interspecific matings with male *martinii* and male *palpalis*.

It was, however, possible to overcome this difficulty by two methods. (a) By cutting off the points of the male superior claspers from the living fly under a binocular microscope; great care has to be exercised that the membrane between the two superior claspers is not damaged, otherwise the body fluid escapes and results in the death of the fly. (b) By carefully painting the underside of the female abdomen with a protective paint or preparation; celluloid dissolved in amyl acetate was found to be a suitable preparation and successfully protected the female abdomen.

Normally, male *martinii* have far larger superior claspers than male *fuscipes*, and male *palpalis* smaller ones than *fuscipes*. Nevertheless, male *palpalis* kill a percentage of female *fuscipes* with which they are mated. It has been possible to breed first and second generation *martinii* males in the laboratory, and, as is usual with laboratory-bred flies, they are smaller individuals and have relatively smaller claspers. Several F₁ and F₂ male *martinii* were

mated with numerous female *fuscipes*. These males were shown at death to have smaller superior claspers than the average found in male *fuscipes*, yet they invariably injured the females and caused their death. This phenomenon has been studied by killing pairs *in cop.* and sectioning them. Owing to relative differences in the size of the superior claspers, in the length of the eighth segment, and in the positions of the juxta and inferior claspers, these males are unable to copulate unless their superior claspers are kept at right angles or pointing towards the anterior end of the female, and presumably their efforts at clasping the female in order to insert the penis result in the claspers piercing the female's abdomen. Neither *G. p. martinii* nor *G. p. palpalis* can successfully copulate with female *G. p. fuscipes*, since the claspers fail to pull the uterus in line and to open the second valve-

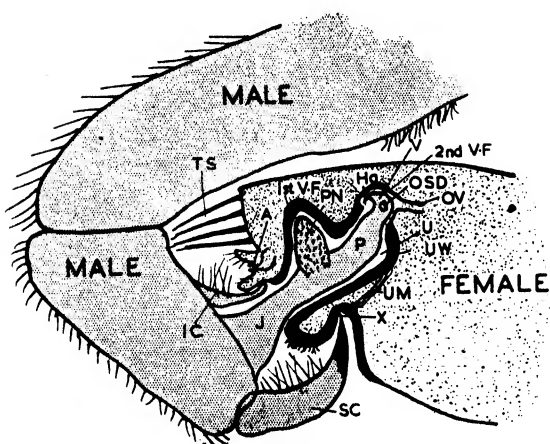


FIG. 1. Normal coitus, *G. tachinoides* × *G. tachinoides*.

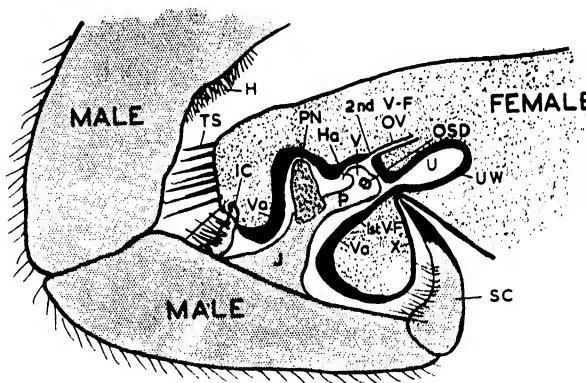


FIG. 2. Abnormal coitus, male *G. p. palpalis* × female *G. p. fuscipes*.

Schematic longitudinal sections of normal and abnormal coitus. The drawings have been prepared from serial sections, and several sections have been used, since the various structures shown do not all occur in the same section. The superior claspers (SC) grip either side of the female abdomen, the inferior claspers or parameres (IC) and the pneumopophyses (PN) lie about midway between the mid-line and the extremities. The juxta (J) and penis (P) are drawn as a whole (not in section), and the mid-longitudinal section is shown of each female.

A.—anus; H.—hectores; Ha.—harpes; IC.—inferior clasper or paramere; J.—juxta; OSD.—opening of the sperm duct; OV.—oviduct; P.—penis; PN.—pneumopophyses; SC.—superior clasper; TS.—terminal or apical spines; U.—uterus; UM.—uterine muscle; UW.—uterine wall; V.—vesica; Va.—vagina; 1st and 2nd V-F.—position of 1st and 2nd valve-folds; X.—normal position for male claspers gripping female.

fold. In these crosses the sperms are discharged into the uterus and presumably make their way into the spermathecae. No exact method of measuring the numbers of sperms in the spermathecae has yet been devised, but females so mated generally appear to have only slightly opaque spermathecae, and consequently a smaller number of sperms, than those normally paired. Fig. 1 shows the normal position of the superior clasper, etc., in intraspecific matings, and fig. 2 the abnormal position in interspecific matings. Although the claspers of male *fuscipes* do not pierce the abdomen of an alien female, they clasp, not in the normal way, but in the same way as *martinii* and *p. palpalis*. The same position is taken up when *palpalis* species are mated with members of the *morsitans* group or when *tachinoides* is mated with *pallidipes*.

3. Failure to Inseminate

The numbers of females proved by dissection at death to be without any sperms in the spermathecae can be seen in Table I by the differences between columns 3 and 4 and the percentage of females inseminated in column 5. These are rather arbitrary figures, since, if the flies did not remain in coitus for 15 minutes or more, they were not considered to have mated and are therefore not included in column 3 ('No. of pairs mated'). Mellanby (1937) has stated that if pairing (for *G. p. fuscipes*) lasted for less than half an hour insemination was unusual, and that if it lasted for half an hour or more the females were generally inseminated. I have had females which have been paired for less than one minute and have reproduced, while others of the same species have been in coitus for 24 hours or more and have apparently not been inseminated. Nevertheless, the frequency of successful inseminations does rise with the duration of pairing: only a few *pallidipes* and members of the *palpalis* and *fusca* groups failed to become inseminated within an hour; *morsitans*, *swynnertoni* and *austeni*, on the other hand, require longer, two hours being necessary to ensure approximately 95 per cent. successful inseminations. Intraspecific crosses of *palpalis* vary from 73 to 82 per cent. successful inseminations, and cross-mated female *palpalis* averaged 86–97 per cent., except when female *fuscipes* were crossed with male *martinii*, which gave only 69 per cent.—but this may be regarded as a special case, on account of the injury caused to the female by the male claspers. The *morsitans*-group controls varied with the species from 60 to 100 per cent., and the cross-matings varied from 50 to 100 per cent. but were better than the corresponding controls. Laboratory-bred controls and hybrids gave very high successful insemination-rates.

Failure to inseminate may be due to the following causes: (a) impotency of the male; (b) psychological effect of captivity; (c) morphological disparity of the genitalia. Cause *a* is rare in males over 10 days old which have not been mated too frequently. It is doubtful whether cause *b* exists in tsetse-flies. Cause *c* is the main barrier to successful mating, but by clipping the claspers this barrier can be overcome in certain crosses.

4. Failure to Fertilize

In Table I the difference between column 6 ('No. inseminated and living 21 days or more') and column 8 ('No. of females producing pupae') gives the numbers of females that failed to become fertilized, or, if fertilized, that aborted their larva. In the controls these numbers are normally a small percentage of the whole, except in the case of *G. m. orientalis*, of which three out of four females failed to reproduce. In the interspecific and intergroup crosses this percentage rises steeply. It has been investigated by taking the mature eggs from the ovaries of virgin females, mixing them with ripe sperm, and incubating the eggs at 27° C. on sterile agar jelly. The results obtained to date by this method are given in Table III and show that fertilization takes place quite readily in most of the interspecific crosses but not in the intergroup crosses. Since it has been possible to rear the eggs only to the hatching stage, it is not known whether the larvae of such crosses as, for example, *pallidipes* with *morsitans*, are capable of full development. As the figures obtained by this method of artificial cross-fertilization are far higher than those obtained by the actual crossing of the species, it seems that some other bar to successful fertilization takes place in the body of the female. This bar may quite possibly be due to (a) inactivation of the sperms in an abnormal environment, (b) difference in pH of the spermathecal ducts, uterus and oviducts, (c) difference in chemotactic attraction, or (d)

failure of larval development owing to ineffective gene combinations. It has already been noted that some females interspecifically mated with certain males all reproduced, whereas others invariably failed to do so ; but even this does not elucidate the problem of whether the sperms of certain males are more amenable to an alien environment or whether the male has a less lethal gene complex.

At present little is known of the chromosomes of *Glossina*. Dr. Slizynski, of Edinburgh, is studying the problem and finds that *G. m. morsitans* has two pairs of V-shaped autosomes and two sex chromosomes, which seems to be true for all the species used in the present breeding-experiments. I had difficulty in finding suitable organs for chromosome work, but this difficulty was overcome by finding large chromosomes in the halteres, which can be examined without undue injury to the fly. The results of studying

TABLE III

Showing the number of eggs taken from virgin female tsetse, mixed with mature male sperms, and incubated on sterile agar jelly at 27° C. ; the number which showed development ; and the percentage fertilized

Female species	Male species	No. of eggs tested	No. which developed	Percentage fertilized
<i>G. p. fuscipes</i>	<i>G. p. fuscipes</i>	10	10	100
"	<i>G. p. martinii</i>	15	14	93
"	<i>G. m. morsitans</i>	15	1	7
"	<i>G. fuscipleuris</i>	5	0	0
"	<i>G. swynnertoni</i>	7	0	0
<i>G. p. martinii</i>	<i>G. p. martinii</i>	8	8	100
"	<i>G. p. fuscipes</i>	12	12	100
"	<i>G. m. morsitans</i>	16	2	12
"	<i>G. fuscipleuris</i>	6	0	0
<i>G. m. morsitans</i>	<i>G. m. morsitans</i>	8	8	100
"	<i>G. swynnertoni</i>	11	10	91
"	<i>G. p. fuscipes</i>	7	0	0
"	<i>G. p. martinii</i>	6	0	0
"	<i>G. pallidipes</i>	8	2	25
"	<i>G. fuscipleuris</i>	9	0	0

hand-fertilized eggs and different crosses would suggest that gene differences prevent intergroup fertilization, but that other causes are responsible for preventing the eggs from becoming fertilized in interspecific crosses.

5. Failure of Development

(a) *Larval Stages*. Except in the later stages it is difficult to tell with certainty whether a female is gravid. Many cross-fertilized females, particularly crosses of *pallidipes* and *morsitans*, have been suspected of being pregnant but no further development has taken place, and, although some of these individuals have been carefully watched, no larvae have been aborted. It may be that in certain crosses larvae had developed, but that, owing to the nature of the cross, a very high mortality-rate was suffered in the larval stages. Such larvae may be reabsorbed and not aborted. With the *G. swynnertoni/submorsitans* cross it was found that first stage and even second stage larvae develop and then die, being either aborted or reabsorbed. It seems that the choriothete may actually digest the larval skins, and may so be able to absorb an early-stage larva. Comparison of the percentage of artificially fertilized eggs and the actual numbers of larvae produced by the crosses

does suggest that it is likely that early development takes place, but that there is a high mortality-rate when the eggs are due to hatch or at some period in the larval stage.

(b) *Pupal Stages*. Table IV compares the viability of pupae from control and cross matings, all pupae having been maintained under identical and comparable conditions. The mortality-rates are generally and significantly higher in pupae from interspecific crosses than in control matings. Some of the data are scanty, but the summation of the results leaves no doubt that the mortality is higher amongst pupae from the interspecifically mated flies.

TABLE IV
Comparison of the viability of pupae from control matings and cross matings

Reference no. of species	Species mated Male Female	No. of full-sized pupae produced*	No. that emerged	Percentage emergence	Percentage failing to emerge
PALPALIS-GROUP CONTROLS					
PaPa	<i>G. p. palpalis</i> × <i>G. p. palpalis</i>	100	98	98	2
FF	<i>G. p. fuscipes</i> × <i>G. p. fuscipes</i>	40	40	100	0
TT	<i>G. p. martinii</i> × <i>G. p. martinii</i>	44	41	93	7
TaTa	<i>G. tachinoides</i> × <i>G. tachinoides</i>	20	18	90	10
PALPALIS-GROUP CROSSES					
F Pa	<i>G. p. fuscipes</i> × <i>G. p. palpalis</i>	12	4	33	67
T Pa	<i>G. p. martinii</i> × <i>G. p. palpalis</i>	5	2	(40)	(60)
PaF	<i>G. p. palpalis</i> × <i>G. p. fuscipes</i>	3	1	(33)	(66)
T F	<i>G. p. martinii</i> × <i>G. p. fuscipes</i>	15	5	33	66
PaT	<i>G. p. palpalis</i> × <i>G. p. martinii</i>	9	4	44	56
F T	<i>G. p. fuscipes</i> × <i>G. p. martinii</i>	5	3	60	40
MORSITANS-GROUP CONTROLS					
I I	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> ex Kondoa	95	93	98	2
MaMa	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> ex Ankole	3	3	100	0
K K } KWK }	<i>G. m. orientalis</i> × <i>G. m. orientalis</i>	10	9	90	10
S S } D D }	<i>G. swynnertoni</i> × <i>G. swynnertoni</i>	45	42	93	7
KWD } WD }	<i>G. pallidipes</i> × <i>G. pallidipes</i>	18	17	94	6
A A	<i>G. austeni</i> × <i>G. austeni</i>	105	101	96	4
MORSITANS-GROUP CROSSES					
S I	<i>G. swynnertoni</i> × <i>G. m. morsitans</i>	20	14	70	30
K I	<i>G. m. orientalis</i> × <i>G. m. morsitans</i>	20	18	90	10
X I	<i>G. m. submorsitans</i> × <i>G. m. morsitans</i>	3	3	100	0
I Ma	<i>G. m. morsitans</i> × <i>G. m. morsitans</i> ex Kondoa ex Ankole	8	8	100	0
I K	<i>G. m. morsitans</i> × <i>G. m. orientalis</i> ex Kondoa	12†	8	66	33
S K	<i>G. swynnertoni</i> × <i>G. m. orientalis</i>	20	14	70	30
MaX	<i>G. m. morsitans</i> × <i>G. m. submorsitans</i> ex Ankole	7	3	43	57
I X	<i>G. m. morsitans</i> × <i>G. m. submorsitans</i>	1	1	(100)	(0)
I S	<i>G. m. morsitans</i> × <i>G. swynnertoni</i> ex Kondoa	5	4	(80)	(20)
K S	<i>G. m. orientalis</i> × <i>G. swynnertoni</i>	5	3	(60)	(40)

* Less the number which were used in various experiments, such as a study of chromosomes and of pupation hormone.

† Five more larvae were produced but failed to pupate.

6. Reciprocal-Effect Difference

It will be noted that when two subspecies are crossed the reproductivity (i.e., the number of females which reproduced) varies significantly between species: for example, when male *swynnertoni* were crossed with female *m. morsitans* (SI cross) only five in 80, or 6.2 per cent., reproduced, whereas when male *m. morsitans* were crossed with female *swynnertoni* 14 in 59, or 24 per cent., reproduced. It might have been expected that the reproductivity of such crosses would not have varied so greatly from one another. Not all the controls reproduced themselves 100 per cent.: for instance, *swynnertoni* controls gave only 59 per cent. (16 in 27) reproductivity, against 95 per cent. (40 in 42) reproductivity in *m. morsitans*. It is possible to compare these figures theoretically on the basis

TABLE V

Showing the percentage (actual and corrected) of females in various crosses which produced pupae, on the basis that 100 per cent. of the control females reproduced

Species	Actual no.	Uncorrected reproducibility	Corrected reproducibility
MORSITANS GROUP			
II cross	40 in 42	95%	100%
KK "	1 " 4	25%	
SS "	16 " 27	59%	
IS "	5 " 80	6.2%	10.5% } Difference not 14.6% } significant
KS "	3 " 35	8.6%	
SI "	14 " 59	24%	25% } Difference not 32% } significant
KI "	16 " 54	30%	
IK "	6 " 9	67%	100% } Difference not 100% } significant
SK "	11 " 19	58%	
PALPALIS GROUP			
PaPa cross	22 in 24	92%	100%
FF "	22 " 22	100%	No correction
TT "	17 " 17	100%	" "
FPa "	6 " 24	25%	27%
TPa "	3 " 7	43%	47%
PaT "	4 " 18	24%	24%
FT "	5 " 23	22%	22%
PaF "	2 " 45	4.4%	4%
FT "	10 " 20	50%	50%

of the controls being 100 per cent. reproductive. If the SI cross of 6.2 per cent. is multiplied by $\frac{100}{95}$, this should give the figure expected had all the control *swynnertoni* reproduced. Table V shows the reproductivity of crosses for which data are adequate and the corrected figures.

Even after such corrections have been applied there are still marked and significant differences between the two crosses. It is difficult to estimate the exact cause of such differences, and from experiments on artificially fertilized eggs (see Table III) it would appear that a greater percentage is capable of becoming fertilized than is actually repro-

duced by the cross in question. This lowered reproductivity may be due to one or both of the following causes: (a) lowered activity or viability of the sperm in an alien environment; (b) higher mortality of the larval stages of the interspecific crosses, due to genetic differences or to adverse effect of an alien environment in the uterus.

Other causes which prevent *Glossina* from reproducing in the laboratory have already been noted, but further work is necessary before the actual cause of these reciprocal differences can be determined.

The mean reproductivity, expressed as a percentage, is obtained by combining the corrected reproductivity of the different reciprocal crosses; it varies with the relative distribution of the species (see Table VI).

TABLE VI

Combining the reproductivity of the cross and its reciprocal. It is assumed that species which reproduce best together are more nearly related than those which do not reproduce so well

Cross	Mean reproductivity
MORSITANS GROUP*	
I K cross and K I cross	66%
K S " S K "	57%
I S " S I "	18%
I X " X I "	27%
S X " X S "	0%
} Compare these figures with those of Table V, where the females are grouped together	
PALPALIS GROUP†	
T F cross and F T cross	36%
PaT " T Pa "	35½%
PaF " F Pa "	16%
} Difference not significant	

* Each group is significantly different from the other. As expected, the figures for this group suggest that *orientalis* is intermediate between *swynnertoni* and *morsitans* and that *morsitans* is intermediate between *swynnertoni* and *submorsitans*.

† From the results for this group it would appear that *martinii* is intermediate between *palpalis* and *fuscipes*—a possible but unexpected result.

OCCURRENCE OF NATURAL HYBRIDS

Natural hybrids of *G. swynnertoni* and *G. m. morsitans* have been found where the environments of the two species fringe upon and overlap each other. This overlap is not great and is never more than a few miles in depth. There are four areas in Tanganyika Territory where the species meet and overlap: (a) the Buhungukira area in Lake Province; (b) the Nindo area in Shinyanga District; (c) the Chungai area, Kikori, Central Province; and (d) the Babati area in the Central Province. Lloyd (1935) found abnormal flies in the Buhungukira area, which he supposed were possibly hybrids. No data have been collected in the Nindo area, but Vanderplank (1947a) has reported *morsitans-swynnertoni* hybrids in the Babati and Chungai areas. In the Buhungukira and Nindo areas *swynnertoni* are isolated from the main *swynnertoni* fly-bush, and in the Chungai and Babati areas *morsitans* are isolated from the main *morsitans* belt. This is of interest, since, if any new characters were gained from the small percentage of fertile female hybrids, one would expect them in time to spread throughout the whole area, and so render the flies in those areas distinguishable from those found in the main belts. Such an event might even give rise to new species, but, so far, the *morsitans* from Kondoa Irangi (areas c and d) appear typical of those found

in the central *m. morsitans* belt and cross normally with those collected at Ankole, some 400 miles away. Similarly, the *swynnertoni* from Shinyanga appear typical of those found in localities in the main belt. These contacts may already have existed for up to a hundred years, but it is improbable that they have existed longer, since the areas were at one time the main seat of the tribes living in the region.

No other definite hybrids are known, and other species likely to hybridize do not come into contact with one another. A *pallidipes-morsitans* hybrid has been suspected, but so far no definite evidence has been obtained, and laboratory data would suggest that such a hybrid is very unlikely.

Status of G. newsteadi

The position of *G. newsteadi* in relation to *G. caliginea* and *G. pallicera* is an interesting subject for speculation. The characters of *G. newsteadi* are intermediate between *G. caliginea* and *G. pallicera* and the parameres have certain affinities to *G. palpalis*. It is found only in one comparatively small area of the Belgian Congo, and could possibly have arisen through hybridization between *G. caliginea* and *G. pallicera* or *G. palpalis*. It is, however, more likely to be an intermediate evolutionary type which has evolved from *G. caliginea* and *G. pallicera* through geographical isolation of a cline in the distribution of *G. pallicera*. Both *G. caliginea* and *G. pallicera* occur in the coastal regions of West Africa, *caliginea* being found in two isolated belts and *pallicera* in three isolated regions, one of which is the Belgian Congo considerably isolated from the other two. I have been able to examine only a few specimens of these species from the different areas, but the parameres of the male genitalia do vary quite considerably from area to area.

Natural hybrids are most likely to occur in areas where two species meet, and not in localities normally inhabited by both species.

CROSSING OF HYBRIDS

1. *Palpalis Group*

The male and female F_1 hybrids bred from crossing the three subspecies of *G. palpalis* with one another have been crossed between themselves and the parental species. Most of the male hybrids are unable to copulate naturally owing to abnormalities in the relative sizes of their superior claspers, juxta and paramere. Some male hybrids were found to kill the female by piercing the abdomen with their superior claspers. In some cases copulation was possible after the male claspers had been clipped, and in each of these cases the males were found to be fertile. The same difficulty, though to a lesser degree, was experienced with the females, but those which mated successfully were found to be fertile. The offspring of the F_2 generation have mating difficulties, but they are otherwise fertile.

2. *Morsitans Group*

The male F_1 hybrids have all proved to be capable of copulation but are consistently sterile. Little is known of the chromosomes of the hybrids. The males, when they are crossed with other hybrids or back-crossed with parental species, have on every occasion so far proved to be sterile, though F_2 male hybrids, produced by back-crossing female F_1 hybrids with parental species, have proved only partially sterile; one F_2 male hybrid was successfully back-crossed with a parental species.

The female F_1 hybrids are partially sterile (the percentage varies according to the type of cross), being fertile with one or both of the parental species. This type of male sterility and female partial sterility has also been previously noted by workers on *Drosophila* hybrids. The data are summarized in Table I. No hybrids have so far been produced between members of the *fusca* group. Owing to the general difficulties of breeding tsetse in captivity, and to the fact that only a few females produce hybrids, few have been produced to date—too few to estimate the comparative degrees of female sterility between the different types of crosses. A description of the various hybrids produced to date is given by Vanderplank (1948a, 1948c).

STATUS OF SUBSPECIES

1. Palpalis Group

Most workers and systematists on the tsetse-flies have been disinclined to accept Zumpt's *G. p. martinii* as a subspecies of *G. p. palpalis*, on the ground that it is either very similar to or identical with *G. p. fuscipes*. These workers, however, have not studied the two subspecies closely, since Zumpt missed several diagnostic differences between the two. He does not note the constant large relative size of the superior claspers of *martinii* to *fuscipes*, nor the importance of the manner in which the parameres of the two species are bent and folded in the living flies. He based the subspecies mainly on the differences in shape and bristles of the flattened parameres. Experimental work has now shown that when male *martinii* are mated with female *fuscipes* they invariably kill the female; but when male *fuscipes* are mated with female *martinii*, which they do somewhat reluctantly, coitus, though not normally possible, can take place. When these difficulties are overcome, however, either naturally or artificially, fertile offspring is produced. This is also true in the case of *G. p. palpalis* and the other two subspecies. *G. p. fuscipes* is probably isolated by a narrow separation from the rest of *palpalis*, but *G. p. martinii* is probably continuous with *G. p. palpalis*. The breeding-experiments also suggest that *G. p. martinii* is intermediate between the two species (see Table V), which fits in with the known facts of its geographical distribution. Examination of the male genitalia of specimens caught in various localities from all parts of Africa shows that *fuscipes* forms a homologous population around Lake Victoria and in Uganda and the Sudan, but that there is a graduation from the *martinii* form through the Congo to the typical *palpalis* form from Nigeria. Two other varieties or subspecies have also been encountered in the north and south-western extremities of distribution, namely, *G. p. gambiensis* and *G. p. angolensis* (see Vanderplank, 1948c).

The question now arises of the status which should be accorded to these species and so-called varieties, races or subspecies. Since some, and in certain cases all, individuals will attempt to mate with individuals from other areas and can produce fertile offspring, I consider that they are best regarded as subspecies, and have treated them as such.

2. Morsitans Group

In the *morsitans* group we have four closely related tsetse, namely, *G. swynnertoni*, *G. m. orientalis*, *G. m. morsitans* and *G. m. submorsitans*, on which experimental breeding data have been collected. On morphological grounds there are three other possible races, *G. m. congolensis*, *G. m. ugandensis* and *G. m. gambiensis* (Vanderplank, 1948a). *G. austeni* is quite separate and distinctive. *G. pallidipes* is separate from both *morsitans* and *austeni*,

but is very similar to *G. longipalpis* and is most probably merely the eastern form ; but breeding-experiments will be necessary before their relationship can be decided.

In the four closely related *morsitans* we find that (a) they will intermate, though the degree to which they do so varies with the geographical proximity of the species, and they are all capable of copulating with one another and of insemination ; (b) only a percentage of the interspecifically mated females reproduce, this percentage (see Table V) varying with the geographical distribution ; (c) all the male F_1 hybrids are sterile, the females only partially so ; (d) some of the male F_2 back-crossed hybrids are fertile.

There is no cline between *G. swynnertoni* and *G. morsitans*, but, where *G. swynnertoni* is in contact, hybridization occurs. A doubtful cline exists between *G. m. orientalis* and *G. m. morsitans* through Nyasaland, Northern Rhodesia and the Belgian Congo, though it is probably severed in Nyasaland and in Northern Rhodesia ; more detailed surveys, however, are needed. Again, it is extremely doubtful whether *G. m. morsitans* is continuous with the *G. m. submorsitans* of Nigeria or even with *G. m. congolensis* from the Congo. *G. m. ugandensis* is separated from other species. *G. m. submorsitans* is broken up into numerous isolated populations.

The differences in behaviour and physiology of the different *morsitans* are slight, and to give them different specific names would tend to confuse workers in the field. On the other hand, there are definite differences between these belts of *G. morsitans*, which may account for the differences in results obtained by workers in Southern Rhodesia, Tanganyika and Nigeria, all of whom have published the results of their experiments on *G. morsitans*, though in fact they were each working on a different subspecies, namely, *G. m. orientalis* in Southern Rhodesia, *G. m. morsitans* in Tanganyika, and *G. m. submorsitans* in Nigeria. The main reasons for considering them as subspecies and not as full species are (a) that they will intermate and inseminate, with the production of hybrids, of which the females are partly fertile, and (b) the existence—at any rate in comparatively recent times—of a cline between each of the subspecies. *G. swynnertoni* has been left as a separate species, since it is more distinctive and has been geographically separated from the rest of *G. morsitans* for a longer period of time ; but when it is considered in relation to other species it should be regarded as *G. morsitans swynnertoni*.

3. Fusca Group

Very little experimental work has been done on the *fusca* group. The few data that have been obtained tend to show that the flies will not even intermate. The various species of the group have, however, become broken up and geographically isolated ; they would form interesting material for future study. Since the members of the group are generally found only in deep forest, they have at the moment little economic importance.

DISCUSSION

Work to date is insufficient to decide the exact relationship between the various groups and species of *Glossina*. The three groups, however, are quite distinct, though they have intermediate forms. The male genitalia of *G. austeni* are intermediate between *G. palidipes* and *G. tachinoides*, and those of *G. caliginea* (*pallicera* and *newsteadi*) are intermediate between *G. palpalis* and *G. brevipalpis*. The two groups studied to date, with their subspecies, make an interesting comparison of new species being formed by geographical isolation, *morsitans* being more advanced in this respect than *palpalis*. *Palpalis* is confined

to habitats alongside lakes or rivers, and is found almost continuously from West to Central Africa, *G. p. martinii* being over 2,250 miles away from the Nigerian form of *G. p. palpalis*. Jackson (1945) has found that the average movement of *G. morsitans* during the whole of its life is only half a mile in any direction. Since *G. palpalis* is restricted to a linear habitat its movement may be somewhat greater, but it probably does not exceed an average of more than a mile. Hence, if an advantageous mutation occurred in *G. p. martinii*, it would require at least 2,250 generations before it could reach Nigeria. As each generation reproduces in a minimum of seven weeks, over 300 years would elapse before *martinii* could travel across Africa. This estimate is based on several fallacies, however, since it assumes that the mutation is dominant and will spread the maximum distance each generation. Moreover, it is possible that the mutation would travel in the same way as the Brownian movement, in which the distance covered is proportional to the time squared, and, if this were so, then the two extremities of the cline would be separated by some thousands of years. The observed differences between the subspecies are not great, however, and it would appear quite feasible that such differences could arise, under laboratory conditions, from one strain kept segregated in two groups for a period of less than 300 years. In any case, continuous climatic changes are taking place more rapidly than speciation, and parts of the cline are liable to become isolated or able to expand their environment. *Glossina* are restricted to certain types of vegetational habitats, which depend on the amount of annual rainfall. From what is known of the climate of tropical Africa, derived from water-borne deposits and the levels of the various inland lakes, there has been a cycle of wet and dry periods, possibly coinciding with the Ice Ages. The present tendencies are towards a drier period, the effect of which is to isolate widely distributed species into several geographical groups. At the same time, the thorn bush, together with *G. swynnertoni*, has spread and is advancing into territory held by *G. morsitans*. No doubt *G. p. fuscipes* was continuous with the rest of *G. palpalis* up to a few hundred years ago, but now it appears to have become separated. The activities of man are also having a marked effect, such a species as *G. palpalis*, for instance, which is found only in linear distribution, being easily eradicated by extensive clearing-operations and so capable of being isolated. The four areas where *G. swynnertoni* and *G. morsitans* now meet were entirely free from any tsetse until about 50-100 years ago, being, in fact, well-populated native chiefdoms. But the drier climatic conditions and the spread of the thorn bush, without any organized control, drove the people and their cattle away from the advancing bush and tsetse, until the tribes were finally reduced in numbers and the areas abandoned. Present conditions, however, are now reversed, since with organized control both bush and fly are being destroyed while the tribes increase and expand.

Another possible and likely limitation to the advance of any particular species of tsetse is the presence of another species in the neighbouring country. It appears that some species, such as *palpalis* and *morsitans*, are liable to intermate to a small extent; thus *palpalis*, spreading inland in a suitable habitat already occupied by *morsitans*, is liable to intermate, with resulting sterility, and so, provided that each population is of equal strength, to form an effective barrier to further advancement, though a numerically superior population is, of course, able to advance at the expense of the numerically inferior one. In this way large rivers inhabited on their fringes by *G. palpalis* can and do form barriers to *G. morsitans*. On the other hand, some species do not attempt to intermate and are able to share the same habitats.

These observations are of particular interest, since Gause (1934) contends that two similar species with similar ecology cannot live in the same region. Huxley (1940) further elaborates this point, and contends that if the species do not compete then both can inhabit the same environment. *G. m. orientalis*, *G. pallidipes*, *G. austeni* and *G. brevipalpis* are sometimes found in the same habitat. Though they show slight differences in their choice of vegetational types, in their food preferences, breeding-sites and times of optimal activity, all four species overlap very widely in each of these points and can, and do, live in the same habitat. If Gause's contention is correct, then these species are not in competition. Their populations are chiefly limited by predatory action and not by food, and their slight differences in ecology prevent detrimental predatory action against one species giving advantage to another.

Although morphologically distinct (far more so than *G. m. orientalis*, *G. pallidipes* and *G. austeni*), *G. palpalis* and *G. swynnertoni* or *G. morsitans* will intermate to a limited extent, and this form of competition appears to be sufficient to prevent the species from living together in the same habitat. Intermating is due to a slight overlap in the individual variation of wing-beat frequencies of the two groups.

Nash (1937a) considers that climate is the sole factor deciding the distribution of various species. Schwetz (1946), however, is not convinced that distribution is due either to climate or to the distribution of plants, feeling that too little weight has been given to the 'law of geographical distribution,' though he does not explain what he means by the term. Laboratory experiments and experience in the handling of many species of tsetse do not suggest that climate is likely to be the only limiting factor, and from field observations I would suggest that the habits of a species impose a limit on its distribution—for instance, although the habits of the species of *Glossina* are eminently suited to their particular forest habitat, they also make them conspicuous, ill-adapted and an easy prey outside their normal environment.

White (1945) has reviewed the genetic aspect of hybridization and the causes of hybrid sterility, and quotes Haldane's (1922) rule that, when one sex in the offspring of a cross is absent, rare or sterile, that sex is the heterogametic one. White reviews the work on the crossing of various species, subspecies, etc., of *Drosophila*, with its resultant fertile, partly sterile, sterile, and non-production of hybrids, as well as the occurrence of intersexes. (One hybrid intersex or hermaphrodite *Glossina* has already been recorded by Vanderplank, 1945.) White states that:

'Although we have hitherto dismissed hybridization as an effective agent in animal evolution, it is possible that even very rare acts of hybridization may play a certain part, by injecting from time to time a set of foreign chromosomes into the total "stock" of genes possessed by a species. This type of genetic process has been called "introgressive hybridization," and although its significance is difficult to estimate it should not be dismissed. Even if the F_1 hybrids have a fairly low viability and fertility a certain number of the foreign chromosomes or chromosome regions, broken up by repeated crossing-over, may manage to survive, thus increasing the "reservoir" of genetic variability in the population.

'It may be objected that if introgressive hybridization were at all widespread in nature there should be more evidence for it. It must be remembered, however, that the foreign genes will very soon become "diluted," and that after repeated backcrossing their effects in the population will hardly be noticeable on a casual examination.'

This is true of *Glossina* hybrids, the back-crosses of which can be recognized up to the third or fourth filial generation, though not later. The isolated *G. morsitans* in contact with the *G. swynnertoni* belt at Babati and Chungai should form an interesting study in 'introgressive' hybridization, as should also the reciprocal case of isolated *G. swynnertoni* in contact with the *G. morsitans* belt at Shinyanga. Most unfortunately, however, both these areas are marked out for extensive development and tsetse eradication, though possibly other areas, so far unrecorded, where *G. swynnertoni* and *G. morsitans* meet may exist and be available for observations. So far, no morphological, physiological or genetic differences have been detected in *G. swynnertoni* at Shinyanga from those in the main fly-belt, nor in *G. morsitans* from Kondoa Irangi from those in the main *morsitans* fly-belt, although hybrids have been found in the areas of contact. The isolated populations in contact with a closely related species might in time give rise to a third and new species by this process of introgressive hybridization, and the new species would probably have developed an intermating barrier against either of the two parental species and so be able to expand into their territories.

White quotes Dobshansky and Kolter (1939), who found that *Drosophila pseudoobscura* showed an 'aversion' to pairing with *D. miranda*, this aversion being particularly strong in strains of *pseudoobscura* collected in localities near areas where *miranda* occurred. Such a situation can be described as a 'negative cline of crossability,' in contrast to the positive cline which occurs in the series *D. subfunnebris*-*D. m. limpiensis*-*D. m. macrospina* from California to Florida. *G. swynnertoni*-*G. m. orientalis*-*G. m. morsitans*-*G. m. submorsitans* show a positive cline in crossability and productivity, but if *G. m. submorsitans* advanced into Kenya from Uganda, and if *G. swynnertoni* advanced north-eastwards in Kenya, they would show a reluctance to cross when they met, and might be considered as a negative cline in crossability, whereas in actual fact they are the two extremities of a positive crossability cline.

The classification of *Glossina* into three groups, several species and subspecies on differences in the male genitalia is supported by experimental breeding. Newstead originally described *G. submorsitans* as a separate species, later as a subspecies. I prefer to regard it as a subspecies, since a cline or partial (recently severed) cline still exists between it and other subspecies of *morsitans*. Although no breeding-experiments have been carried out, it is doubtful whether *G. pallidipes* and *G. longipalpis* should be classed as separate species instead of as merely subspecies of each other. Breeding-experiments show that minute differences in the genitalia are ample grounds for the formation of subspecies, but that colour-differences are unimportant. Consequently, varieties described on the coloration, such as *G. morsitans* var. *pallida* Shire, *G. morsitans* var. *paradoxa* Shire, *G. palpalis* var. *wellmani* Austen and *G. palpalis* var. *maculata* Newstead have no importance, except in so far as they are useful in studying the genetics of colour-inheritance in *Glossina*.

Individuals collected from different geographical areas in a cline show a relative degree of fertility, which varies with the distance between each locality. The data are scanty, but would suggest that fertility varies directly to distance. The data on this point are summarized in Tables IV and V.

The experiments have brought to light one new subspecies, which can be recognized on morphological grounds. Careful examination of male genitalia suggests that other new subspecies can be recognized by minute but constant differences; but these require confirmation by cross-breeding experiments.

SUMMARY

The data on intergroup and interspecific mating and productivity are summarized in table form and are considered as a whole. There are varied and numerous barriers against intergroup and interspecific crossing, most of which have been encountered in the various types of crosses which have been carried out. They are (a) failure to mate, (b) failure to copulate, (c) damage during copulation, (d) failure to inseminate, (e) failure of the sperms to fertilize, and (f) failure of development of the larval and pupal stages. The sterility or partial sterility of hybrids is discussed. The reciprocal cross is approximately twice (or only half) as productive as its counterpart.

Natural hybrids have been found in the field, and others may exist.

Male *palpalis* hybrids are generally unable to mate successfully, but are fertile when enabled to do so artificially. The female hybrids are fertile.

Male *morsitans* hybrids are sterile, but are able to copulate and to inseminate the females. The female hybrids are partially sterile, but the percentage sterile depends upon the cross.

The status of the various subspecies is discussed in the light of the present work, and it is concluded that experimental work supports the existing classification, with one or two minor adjustments.

The evolution of new species is discussed.

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MALARIA AND BLACKWATER FEVER IN MACEDONIA AND THRACE IN RELATION TO DDT

BY

HENRY FOY

ATHENA KONDI

(Wellcome Trust Research Laboratories, Salonika)

CHRISTOS DAMKAS

MARGARET DEPANIAN

THERESA LEFCOPOULOU

(Macedonia Malaria Services)

L. G. BACH, R. DAX, J. PITCHFORD, PAMELA SHIELE

AND MOIRA LANGTON

(United Nations Relief and Rehabilitation Administration)

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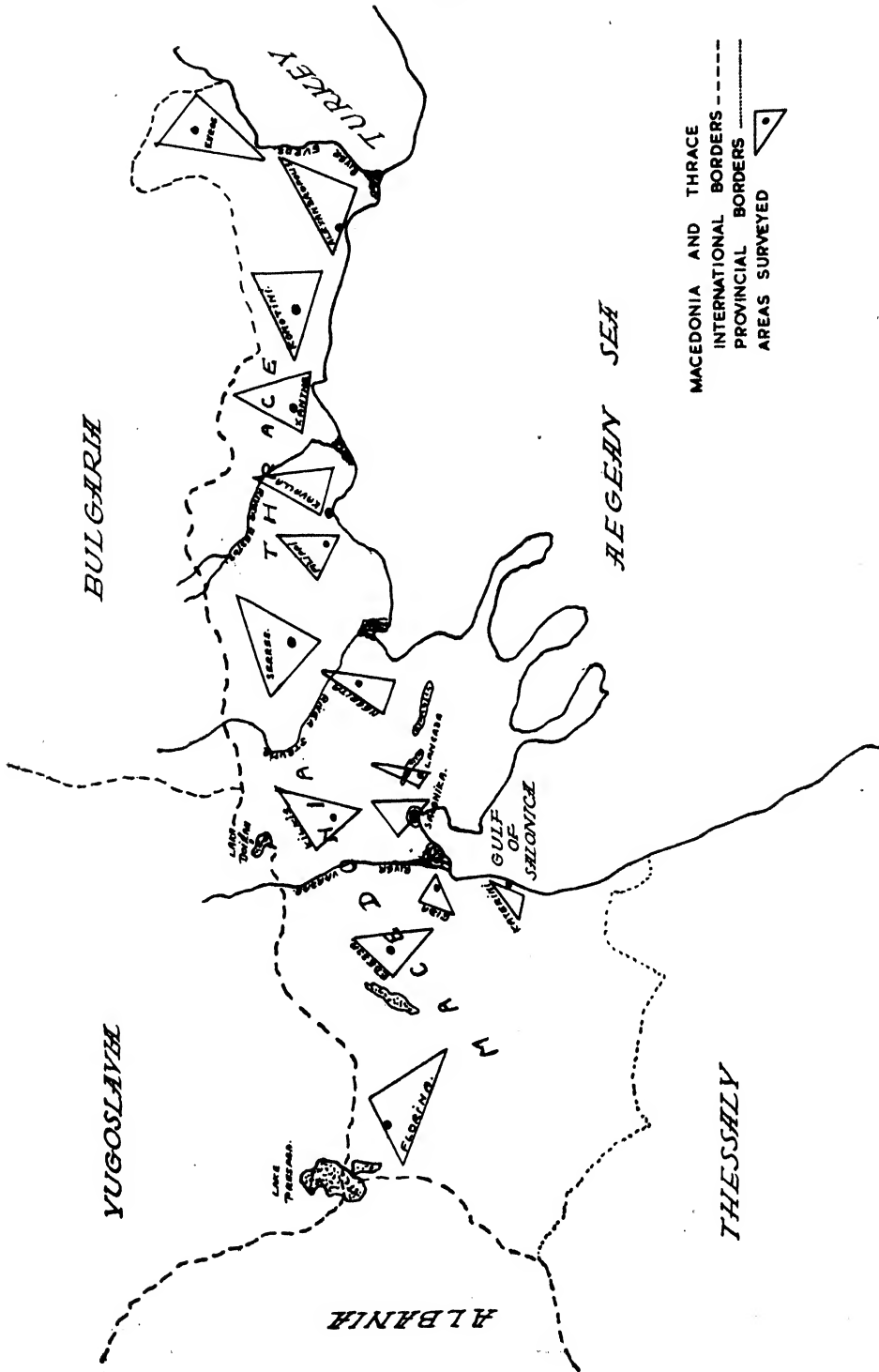
I. INTRODUCTION

The purpose of the present paper is to give the results of a malaria survey carried out in Macedonia and Thrace during November and December, 1946. The object of the survey was to assess the relative effects of (i) the nation-wide DDT campaign of late 1945 and 1946, and (ii) the natural variations in malaria endemicity on the low malaria-rates that have prevailed in northern Greece since the great epidemic of 1942. No attempt is made to discuss the situation in southern Greece.

The difficulties of interpreting the results of any antimalarial measures in a country such as Macedonia or Thrace, where wide natural variations in malaria incidence occur from year to year, have been stressed by Barber and his colleagues (1935, 1936) and by Balfour (1935). These difficulties are especially marked if the antimalarial measures happen to have been started during a period of low transmission. As Hackett (1937) has aptly pointed out, 'if we happen to dip our bucket into the sea of malaria endemicity when the tide is on the ebb, we and the moon together can lower its level in a miraculous way.' The malaria worker must, therefore, beware of attributing to his own efforts results which are properly due to other factors, and, if the incidence happens to be falling steeply when the work commences and no controls are available, he must be particularly critical of his results.

In order, therefore, to appraise the malaria situation of Macedonia and Thrace in 1945 and 1946 it has been necessary to review the position against the background of previous years. In doing this it has been possible to draw upon the large amount of accurate data collected and analysed by teams of previous workers who have investigated malaria in northern Greece, notably the discriminating work of Barber *et al.* (1935-36), of Balfour (1935), and of Livadas and his colleagues (1941, 1946).

It is unfortunate that no areas in Macedonia and Thrace were specially set aside as control regions when it was decided to undertake malaria control by means of DDT. There are, however, certain regions where DDT-treatment was either not carried out at



all or was done in such a way as to render its use of very questionable value, and it is these areas which have been used as a control region.

The DDT campaign was commenced late in 1945, but it was not regarded as being in full swing until the spring and summer of 1946, and even then it was contending with transport and communication difficulties consequent upon the chaos in Macedonia and Thrace brought about by the war. The campaign was directed partly against larvae, partly against adults, in houses and stables, with the emphasis on adult eradication. Spraying from aeroplanes was used against breeding-grounds in areas where conditions permitted.

The main vectors of malaria in Greece are *Anopheles superpictus* and *A. saccharovi*, the former being the more important in mountain areas and in regions of running streams, the latter in plain areas where there are bodies of standing water of varying degrees of salinity. *A. maculipennis* is abundant in certain areas and can easily be infected in the

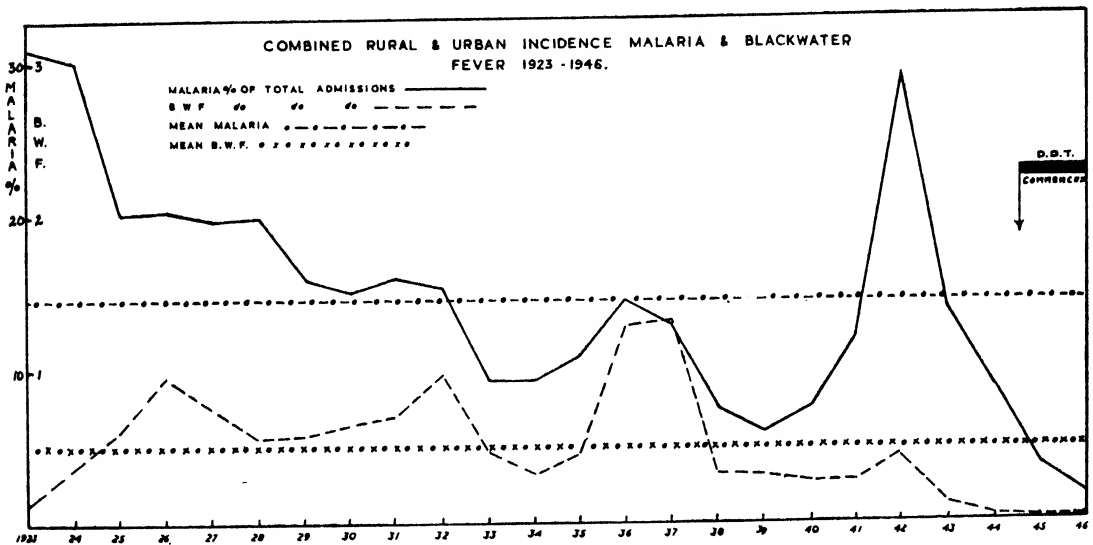


FIG. 1

laboratory, but it has a high degree of animal deviation and its sporozoite-rate is only 0·06 per cent. The sporozoite-rate of *A. saccharovi* and *A. superpictus* is between 1·5 and 3·0 per cent., though in rare instances it may reach a level of 10 per cent. in certain hyper-endemic areas. In the early and high summer the chief vector is *A. saccharovi*, *A. superpictus* becoming abundant in the late summer and continuing transmissions in the later part of the season.

II. THE MALARIA SITUATION IN 1923-34 AND IN 1935-40

In Table I and fig. 1 figures are given showing the variations in malaria incidence in Macedonia during the 24-year period from 1923 to 1946. These figures are drawn from the two hospitals in Salonika with which the Wellcome Trust Research Laboratories have been closely associated since 1935. Diagnosis of malaria was made both clinically and parasitologically. The Central Refugee Hospital is mainly devoted to rural admissions,

the Municipal Hospital to admissions from the urban area of Salonika. The figures and the graph show clearly the periodic fluctuation in malaria incidence in this region. It appears from the work of Barber and his colleagues that these variations in malaria incidence are probably connected with anopheline density, which is dependent upon the rainfall conditions prevailing in the late winter and early spring.

From figs. 1, 2 and 3 it will be seen that the general trend of malaria incidence during

TABLE
Malaria and blackwater fever incidence in Macedonia during the 24-year period 1923-46

Year	Central Refugee Hospital (rural)						Municipal Hospital (urban)		
	Total admissions	Malaria admissions	Blackwater fever admissions	Percentage of malaria in total admissions	Percentage of B.W.F. in total admissions	Percentage of B.W.F. in malaria admissions	Total admissions	Malaria admissions	Blackwater fever admissions
1923	3,409	1,445	3	42.5	0.09	0.20	3,014	489	6
1924	4,491	1,877	11	41.8	0.25	0.59	2,892	349	13
1925	3,821	1,253	21	33.0	0.54	1.67	2,956	203	14
1926	4,637	1,562	52	33.7	1.12	3.32	3,544	318	22
1927	4,608	1,316	43	28.6	0.94	3.26	3,688	322	17
1928	5,092	1,370	37	26.9	0.73	2.70	3,583	385	10
1929	5,324	1,227	40	23.1	0.75	3.27	3,825	291	11
1930	5,291	1,120	59	21.1	1.11	5.30	4,734	394	6
1931	5,014	1,168	54	23.3	1.10	4.60	6,482	736	26
1932	5,413	1,018	76	18.8	1.40	7.46	7,431	974	45
1933	4,723	461	32	9.76	0.68	6.95	7,494	610	23
1934	4,751	491	23	10.3	0.49	4.68	7,478	579	12
Totals	56,574	14,308	451	25.4	0.79	3.16	57,121	5,650	205
1935	5,396	675	43	12.5	0.79	6.37	6,946	718	5
1936	4,958	814	91	16.4	1.84	11.20	6,594	862	53
1937	3,702	613	75	16.5	2.02	12.20	5,996	715	50
1938	4,164	379	18	9.1	0.43	4.80	5,787	367	13
1939	4,637	374	19	8.1	0.41	5.08	5,334	231	13
1940	1,997	247	13	11.8	0.65	5.25	4,888	272	5
1941	1,604	263	1	16.4	0.06	0.38	3,841	395	14
1942	3,241	1,270	7	39.4	0.22	0.55	5,951	1,361	29
1943	3,373	639	3	18.9	0.09	0.47	5,952	619	7
1944	2,984	369	1	12.4	0.34	0.27	6,388	402	2
1945	3,493	183	0	5.2	—	—	7,197	171	2
1946	4,185	89	0	2.1	—	—	6,026	79	2
Totals	43,734	5,915	271	13.5	0.62	4.57	70,900	6,192	195
	56,574	14,308	451	25.4	0.79	3.16	57,121	5,650	205
Grand totals	100,308	20,223	722	20.1	0.72	3.56	128,021	11,842	400

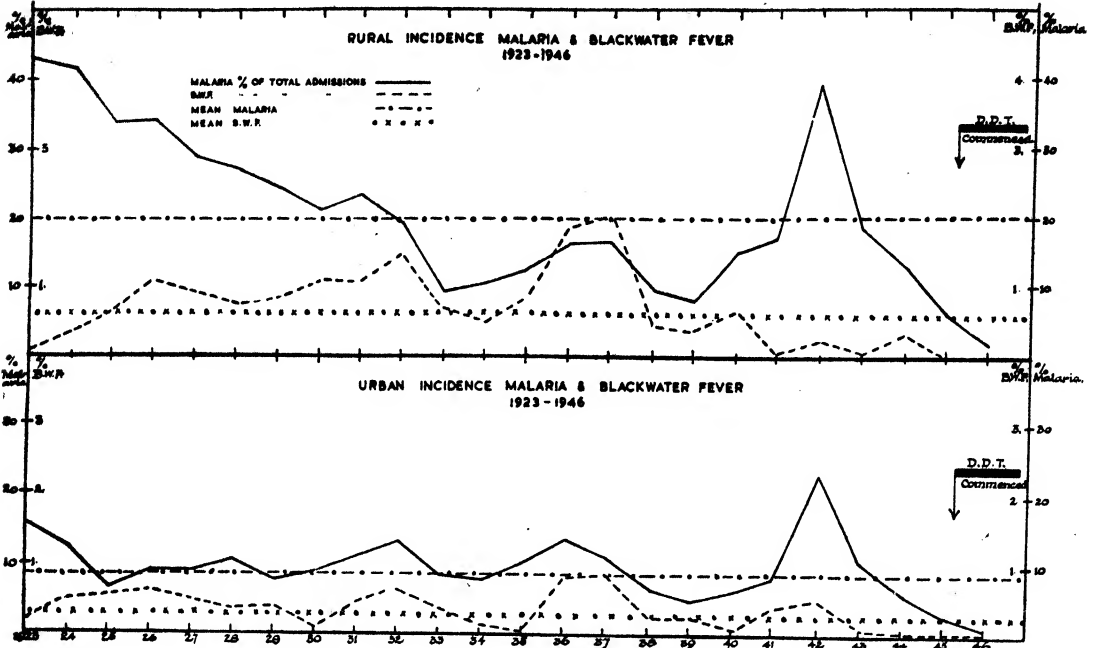
only rising above the mean in the great epidemic of 1942. In the urban areas the incidence has remained more or less stable during the whole of the 24 years, and for most of the period is above the mean rate. The mean incidence for the rural areas fell from 25.4 per cent. in the 1923-34 period to 13.5 per cent. in 1935-46. In the urban areas, on the other hand, the rate remained fairly stable, being 9.8 per cent. during the 1923-34 period, changing to 8.6 per cent. in 1935-46.

Municipal Hospital (urban)			Combined Central and Municipal Hospitals					
Percentage of malaria in total admissions	Percentage of B.W.F. in total admissions	Percentage of B.W.F. in malaria admissions	Total admissions	Malaria admissions	Blackwater fever admissions	Percentage of malaria in total admissions	Percentage of B.W.F. in total admissions	Percentage of B.W.F. in malaria admissions
16.2	0.19	1.2	6,423	1,934	9	30.0	0.14	0.5
12.4	0.45	3.7	7,383	2,226	24	30.2	0.33	1.1
6.9	0.48	6.9	6,777	1,456	35	21.4	0.52	2.4
8.9	0.63	6.9	8,181	1,880	74	23.0	0.91	3.9
8.8	0.46	5.3	8,296	1,638	60	19.8	0.73	3.7
10.7	0.28	2.6	8,675	1,755	47	20.2	0.54	2.7
7.6	0.29	3.8	9,149	1,518	51	16.5	0.56	3.4
8.3	0.13	1.5	10,025	1,514	65	15.1	0.65	4.3
11.3	0.40	3.5	11,496	1,904	80	16.5	0.69	4.2
13.1	0.60	4.6	12,844	1,992	121	15.5	0.95	6.1
8.1	0.31	3.8	12,217	1,071	55	8.8	0.45	5.1
7.7	0.16	2.1	12,229	1,070	35	8.8	0.29	3.3
9.85	0.36	3.64	113,695	19,958	656	17.6	0.58	3.3
10.3	0.07	0.7	12,342	1,393	48	11.4	0.39	3.5
13.1	0.80	6.1	11,552	1,676	144	14.5	1.25	8.6
11.9	0.84	7.0	9,698	1,328	125	12.9	1.29	9.4
6.3	0.23	3.6	9,951	746	31	7.5	0.31	4.1
4.3	0.24	5.6	9,971	605	32	6.1	0.32	5.3
5.6	0.13	1.8	6,885	519	18	7.4	0.26	3.5
7.7	0.36	3.5	5,445	658	15	12.2	0.28	2.3
22.9	0.49	2.1	9,192	2,631	36	28.4	0.39	1.4
10.5	0.12	1.1	9,325	1,258	10	13.7	0.11	0.8
6.4	0.03	0.5	9,372	771	3	8.3	0.03	0.4
2.5	0.02	1.2	10,690	354	2	3.3	0.02	0.6
1.3	0.03	2.5	10,211	168	2	1.7	0.02	1.2
8.65	0.278	3.14	114,634	12,107	466	10.6	0.41	3.9
9.85	0.36	3.64	113,695	19,958	656	17.6	0.58	3.3
9.20	0.31	3.4	228,329	32,065	1,122	14.1	0.49	3.5

the past 24 years has been downward, with 'flare-ups' occurring from time to time. The mean malaria incidence for the whole of the 24-year period was 14.1 per cent.; in the 12-year period from 1923 to 1934 it was 17.5 per cent., and in the 12-year period from 1935 to 1946 it had dropped to 10.6 per cent. The urban incidence of malaria (fig. 3) was about half that of rural areas (fig. 2) in both the 12- and the 24-year periods. Further, it will be seen that the reduction in malaria has taken place mostly in the rural areas. The figures for the rural areas have been below the mean for the whole 24-year period since 1933,

The same difference in urban and rural incidence is found in the case of blackwater fever, where the urban rate was 0.3 per cent. and the rural 0.7 per cent. There is, however, no difference between the percentages of malaria cases that developed blackwater fever in the urban and in the rural districts, being 3.4 per cent. and 3.6 per cent. respectively, thus indicating that the 'blackwater-fever producing capacity' of rural malaria is no greater than that of urban malaria, but that weight of malaria endemicity is probably of importance.

RURAL INCIDENCE MALARIA & BLACKWATER FEVER 1923-1946



Figs. 2 and 3

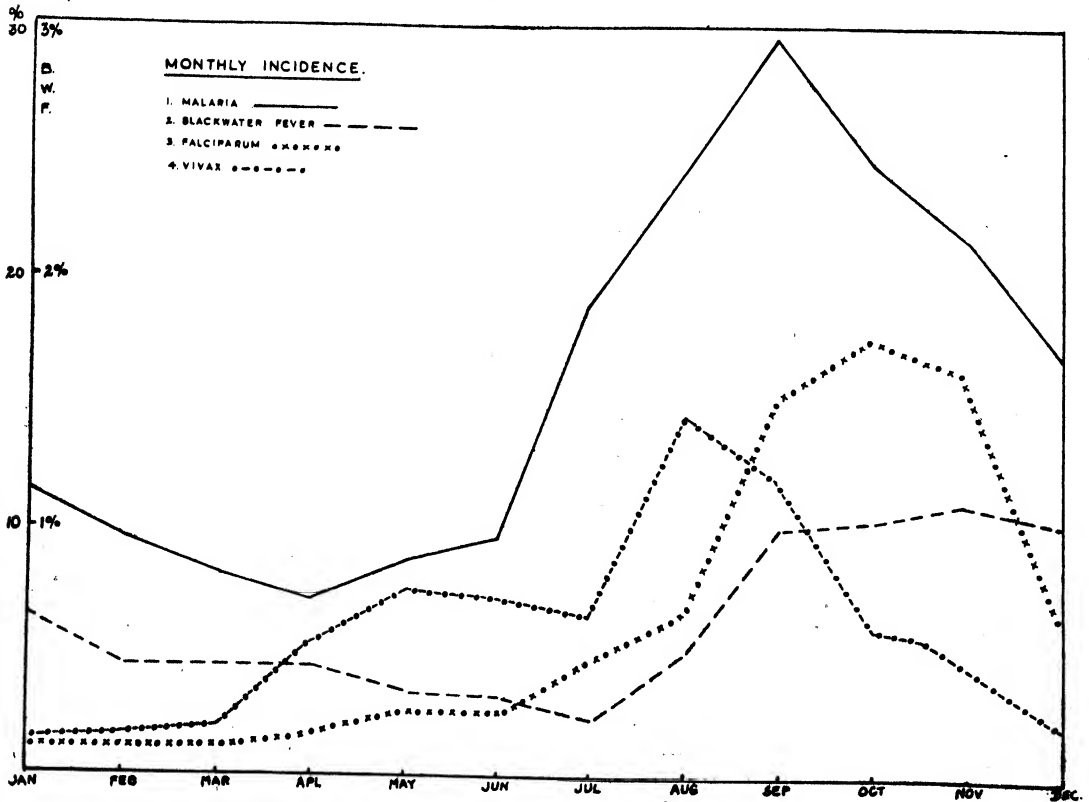


FIG. 4

The monthly incidence of malaria and of blackwater fever, as well as the distribution of *Plasmodium falciparum* and of *P. vivax*, is shown in fig. 4. These have already been analysed in a previous paper (Foy and Kondi, 1938) and need not be commented on further here.

In the late spring and early summer *P. vivax* is the predominant parasite, *P. falciparum* occurring later in the summer and reaching its maximum in August and September. Since infection with *P. falciparum* is usually short-lived, variations in the relative or absolute amount of *falciparum* give some indication of the degree of transmission which is occurring. In seasons of high transmission *P. falciparum* is the predominant parasite, accounting for as much as 70–80 per cent. of the active infections. The parasite-index in Greece is lowest in the February–March period and highest in August–October. The spleen-index in Greece is generally about twice the parasite-index, but this relation is subject to variations during periods of epidemicity and low endemicity (see below). The spleen-index can be taken as a measure of the malarial situation which has prevailed during the previous two or three years. The ratio of the spleen- and blood-index can be used as a measure of the malaria transmission occurring during any given season, and, taken with the parasite-index of infants who have lived throughout only one transmission season, will give a fair indication of the volume of transmissions occurring during any given season.

Apart from periodical rises and falls, there was no major wide-spread epidemic in northern Greece during the period 1930–40, though there was a 'flare-up' in 1935–36, which in some areas of Macedonia reached epidemic proportions (Barber *et al.*, 1936). It may be said that during the period 1925–40 nothing occurred to disturb the general downward trend of malaria incidence, shown in fig. 1, after the settlement of the refugees from Asia Minor in 1923–25.

III. THE MALARIA SITUATION IN 1940–46

In 1942 there occurred a major malaria epidemic, which appears to have affected the whole of Greece, as well as Macedonia and Thrace. The extent of the rise in malaria incidence in Macedonia can be seen in Table I and fig. 1, the incidence jumping from 12.2 per cent. in 1941 to 28.4 per cent. in 1942. In the rural districts (fig. 2) it rose

TABLE II
Combined malaria and blackwater fever death-rates, 1940–46

Year	Total admissions	Malaria admissions	Black-water fever admissions	No. of malaria deaths	No. of blackwater fever deaths	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions	Percentage of blackwater fever deaths in blackwater fever admissions	Percentage proportionate mortality from malaria
1940	6,885	519	18	19	6	0.3	3.7	33.0	4.0
1941	5,445	658	15	25	2	0.5	3.8	13.0	4.9
1942	9,192	2,631	36	147	12	1.6	5.6	33.0	15.9
1943	9,325	1,258	10	16	3	0.15	1.1	30.0	3.5
1944	9,372	771	3	6	1	0.06	0.8	33.0	1.0
1945	10,690	354	2	1	0	0.02	0.6	0.0	0.2
1946	10,211	168	2	3	1	0.03	1.8	50.0	0.7
Totals	61,120	6,359	86	217	25	0.36	3.4	29.0	5.5

TABLE
Death-rate from malaria in

Age-group	Central Refugee Hospital (rural)						Municipal		
	Total admissions	Malaria admissions	Malaria deaths	Percentage of malaria in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions	Total admissions	Malaria admissions	Malaria deaths
1-5	687	73	7	10.6	1.0	9.6	980	63	9
6-10	1,043	161	8	15.4	0.76	4.9	1,874	141	4
11-15	2,161	317	1	14.7	0.05	0.3	3,342	267	11
15+	16,986	2,509	68	14.8	0.39	2.7	34,047	2,828	109
Totals ...	20,877	3,060	84	14.7	0.4	2.7	40,243	3,299	133

from 16.4 per cent. in 1941 to 39.4 per cent. in 1942, and in the urban areas (fig. 3) from 7.7 per cent. to 22.9 per cent. The profound changes which occurred in malaria morbidity during 1941-43 are reflected in the malaria mortality figures shown in Tables II, III and IV. Following Balfour (1935), we have used proportionate mortality (fig. 5), as being the most reliable index for countries where accurate population figures are not available.

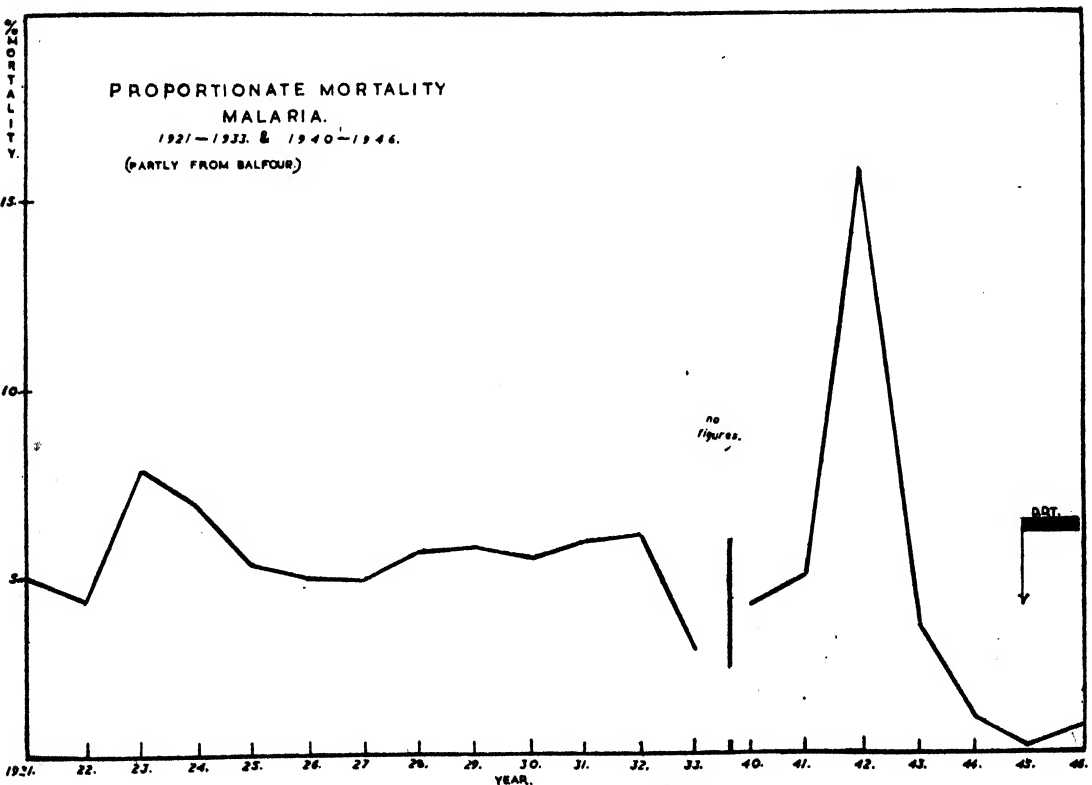


FIG. 5

age-groups, 1940-46

Urban (urban)			Combined					
Percentage of malaria deaths in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions	Total admissions	Malaria admissions	Malaria deaths	Percentage of malaria in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions
0.9 0.2 0.3 0.3	0.9	11.1	1,667	136	16	8.1	0.96	10.3
	0.2	2.8	2,917	302	12	10.4	0.41	4.0
	0.3	4.1	5,503	584	12	10.6	0.22	2.1
	0.3	3.8	51,033	5,337	177	10.4	0.34	3.3
	0.3	4.0	61,120	6,359	217	10.4	0.35	3.4

It will be seen that the proportionate mortality from malaria rose phenomenally from 4.9 per cent. in 1941 to 15.9 per cent. in 1942, and then sank in 1943 to one-fifth of the previous year's figure; during 1944, 1945 and 1946 the fall continued, but, as in the case of morbidity, at a diminishing rate.

Until more information is available it will not be possible to give precise reasons for this great increase. No doubt some of the factors operating were the German occupation and the general break-down of organized life and the starvation which followed. During this period of turmoil there was a wide-spread movement of population between the towns and villages and rural areas, villagers quartering themselves in the towns, and townspeople migrating to rural areas for food and safety, often under primitive conditions in highly malarious regions.

A further factor may have been the loss of immunity of the population following a number of years of low malaria transmission. There is no evidence that the absence of quinine was not made good by adequate supplies of atabrin, so that this is not likely to have been a factor. Although no precise data are available, it seems to be generally agreed that there was an increase in the density of *A. superpictus* during the transmission season of 1942. Unfortunately, no figures are available for sporozoite-rates, mosquito-density or rainfall. Nor were spleen- or blood-indices taken during that year in northern Greece. There is, however, general agreement that malaria reached epidemic levels in 1942—a fact borne out by the figures given in this paper.

Whatever may have been the causes of the epidemic, there followed a dramatic fall in malaria incidence and mortality in 1943, the figures dropping from 28.4 per cent. in 1942 to 13.7 per cent. in 1943 in the combined rural and urban areas. In the urban areas the fall was from 22.9 per cent. to 10.5 per cent. and in the rural areas from 39.4 per cent. to 18.9 per cent. There was a further steep drop in 1944-45 from 8.3 per cent. to 3.3 per cent. in the combined areas; in the rural areas the figures fell from 12.4 per cent. to 5.2 per cent., and in the urban areas from 6.4 per cent. to 2.5 per cent. In previous years neither the rises nor the falls were so steep.

The reasons for the unusually steep fall are probably complex and may be related to a number of factors, of which the substitution of atabrin for quinine may have been one. In the Pacific campaign of the recent war the use of atabrin as a causal prophylactic proved its value so far as *P. falciparum* was concerned, and it is known that during epidemics

TABLE
Yearly death-rate from malaria

different age-groups, 1940-46

Year	Age-group	Central Refugee Hospital						Municipal Hospital						Combined					
		Total admissions	Malaria admissions	Malaria deaths	Percentage of malaria in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions	Total admissions	Malaria admissions	Malaria deaths	Percentage of malaria in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions	Total admissions	Malaria admissions	Malaria deaths	Percentage of malaria in total admissions	Percentage of malaria deaths in total admissions	Percentage of malaria deaths in malaria admissions
1940	1-5	139	14	4	10.0	2.9	28.6	172	10	0	5.8	0.0	0.0	311	24	4	7.7	1.3	16.7
	6-10	115	19	2	16.5	1.7	10.5	325	13	0	4.0	0.0	0.0	440	32	2	7.3	0.5	6.3
	11-15	175	30	1	17.1	0.6	33.3	530	34	1	6.4	0.2	2.9	705	64	2	9.1	0.3	3.1
	15+	1,568	184	6	11.7	0.4	3.3	3,861	215	5	5.6	0.1	2.3	5,429	399	11	7.4	0.2	2.8
	Total	1,997	247	13	12.4	0.7	5.3	4,888	272	6	5.5	0.1	2.2	6,885	519	19	7.5	0.3	3.8
1941	1-5	33	4	2	12.1	6.0	50.0	111	8	3	7.2	2.7	37.5	144	12	5	8.3	3.5	41.7
	6-10	75	12	0	16.0	0.0	0.0	222	31	1	14.0	0.5	3.3	297	43	1	14.5	0.3	2.3
	11-15	166	26	0	15.7	0.0	0.0	329	48	5	14.6	1.5	10.4	495	74	5	14.9	1.0	6.7
	15+	1,330	221	4	16.6	0.3	1.8	3,179	308	10	9.7	0.3	3.2	4,509	529	14	11.7	0.3	2.7
	Total	1,604	263	6	16.4	0.4	2.3	3,841	395	19	10.3	0.5	4.8	5,445	658	25	12.1	0.5	3.8
1942	1-5	54	21	0	38.9	0.0	0.0	112	28	4	25.0	3.5	14.3	166	49	4	20.6	2.4	8.2
	6-10	148	66	4	44.5	2.7	6.1	210	52	2	24.8	0.95	3.8	358	118	6	32.9	1.7	5.1
	11-15	300	108	0	36.0	0.0	0.0	406	98	4	24.0	1.0	4.1	706	206	4	29.2	0.6	1.9
	15+	2,739	1,075	50	39.0	1.8	4.6	5,223	1,183	83	22.6	1.6	7.0	7,962	2,258	133	28.4	1.7	5.9
	Total	3,241	1,270	54	39.2	1.7	4.3	5,951	1,361	93	22.9	1.6	6.8	9,192	2,631	147	28.6	1.6	5.6
1943	1-5	57	13	1	22.8	1.75	7.7	79	1	0	1.7	0.0	0.0	136	14	1	10.3	0.7	7.1
	6-10	133	28	1	21.0	0.8	3.6	199	15	0	7.5	0.0	0.0	332	43	1	2.9	0.3	2.3
	11-15	367	75	0	20.4	0.0	0.0	410	30	1	7.3	0.24	3.3	777	105	1	13.5	0.1	0.95
	15+	2,816	523	5	18.6	0.2	1.0	5,264	573	8	10.8	0.2	1.4	8,080	1,096	13	13.5	0.2	1.2
	Total	3,373	639	7	18.9	0.2	1.1	5,952	619	9	10.4	0.15	1.5	9,325	1,258	16	13.5	0.2	1.3
1944	1-5	69	6	0	8.7	0.0	0.0	119	3	0	2.5	0.0	0.0	188	9	0	4.8	0.0	0.0
	6-10	115	13	1	11.3	0.9	7.7	309	15	0	4.9	0.0	0.0	424	28	1	6.6	0.2	3.6
	11-15	273	38	0	13.9	0.0	0.0	557	28	0	5.0	0.0	0.0	830	66	0	8.0	0.0	0.0
	15+	2,525	312	2	12.3	0.1	0.6	5,403	356	3	6.6	0.1	0.8	7,930	668	5	8.5	0.1	0.7
	Total	2,982	369	3	12.4	0.1	0.8	6,388	402	3	6.3	0.05	0.7	9,372	771	6	8.2	0.06	0.8
1945	1-5	140	8	0	5.7	0.0	0.0	188	2	0	1.1	0.0	0.0	328	10	0	3.0	0.0	0.0
	6-10	187	17	0	9.0	0.0	0.0	372	10	1	2.7	0.3	10.0	559	27	1	4.8	0.2	3.7
	11-15	388	24	0	6.0	0.0	0.0	664	22	0	3.3	0.0	0.0	1,052	46	0	4.4	0.0	0.0
	15+	2,778	134	0	4.8	0.0	0.0	5,973	137	0	2.3	0.0	0.0	8,751	271	0	3.1	0.0	0.0
	Total	3,493	183	0	5.3	0.0	0.0	7,197	171	1	2.4	0.01	0.6	10,690	354	1	3.3	0.01	0.3
1946	1-5	195	7	0	3.6	0.0	0.0	199	11	2	5.5	1.0	18.2	394	18	2	4.6	0.5	11.1
	6-10	270	6	0	2.2	0.0	0.0	237	5	0	2.1	0.0	0.0	507	11	0	2.2	0.0	0.0
	11-15	492	16	0	3.3	0.0	0.0	446	7	0	1.6	0.0	0.0	938	23	0	2.5	0.0	0.0
	15+	3,228	60	1	1.8	0.0	0.0	5,144	56	0	1.1	0.0	0.0	8,372	116	1	1.4	0.0	0.0
	Total	4,185	89	1	2.1	0.0	0.0	5,026	79	2	1.6	0.04	2.5	10,211	168	3	1.6	0.02	1.2

in Greece there is an increase of *P. falciparum* infections. Moreover, atabrin was used extensively in Greece during the period of the German occupation, and it is therefore not improbable that its use may have been a factor in the sharp fall in malaria incidence which occurred after the 1942 epidemic. That this may have been so is further suggested by the

fact that, although the rise in malaria incidence was one of the highest on record, the rise in blackwater fever was minimal, changing only from 0.28 per cent. in 1941 to 0.39 per cent. in 1942, compared with a rise from 0.39 per cent. in 1935 to 1.29 per cent. in 1936, when the malaria incidence rose only from 11.4 per cent. to 14.5 per cent. Since the

correlation coefficient between the last dose of quinine and the first passage of black urine is + 0.87 (Foy and Kondi, 1937), it would appear that the relation between blackwater fever and quinine is due to something more than chance. Fairley (1945) has commented on the rareness of blackwater fever in the Pacific campaign, and has suggested that it may be connected with the fact that atebrin eliminates *P. falciparum* infection. Cases of blackwater fever have, however, been reported after atebrin (Foy and Kondi, 1937; Ciuca, quoted by Strong, 1945; Manson-Bahr, 1940; Abbott, 1946), and it is not impossible that any drug useful against malaria may be capable of precipitating blackwater fever. Nor can it be affirmed with certainty that *P. falciparum* is the only parasite concerned in the genesis of blackwater fever (Foy and Kondi, 1938; Langen and Lichtenstein, 1936). Other factors concerned in the low incidence of blackwater fever in Macedonia and Thrace since 1942 will be discussed in detail in a later paper.

A further factor contributing to this steep fall in malaria incidence may have been the acquirement of a high immunity following the 1942 epidemic, as well as the high infant mortality-rate of 1942 and 1943 (Valaoras, 1946), which would have reduced the gametocyte reservoir to a low level.

It does not appear that DDT can be regarded as having played any part in the fall in malaria incidence which occurred in 1943, 1944 and 1945, since it did not come into use until late in 1945. Although malaria has continued to fall during 1945 and 1946 it is doing so at a steadily diminishing rate, as will be seen from the accompanying figs. Whether the fall in 1945 and 1946 can be attributed to DDT is quite impossible to say until more data are available and more years have elapsed in which to judge the situation. As pointed out above, the fact that the DDT campaign started at a time when the malaria incidence was falling rapidly makes it impossible to assess the part which it played in bringing about the low malaria incidence that has characterized the years 1945 and 1946.

IV. THE 1946 SCHOOLS SURVEY

The winter survey of 1946 was carried out in November and December; 11,905 spleens of school-children between the ages of 4 and 14 years were examined, together with 7,331 bloods. In addition, 2,207 bloods were taken from infants under one year old who had lived through only the one transmission season of May–November, 1946.* Blood was taken from the finger-tip, and thick and thin smears were made on the same slide, the former being stained with Giemsa and the latter with Leishman. The slides were examined by a competent team of technicians, who have been engaged on this work for the past 12 years. All spleen examinations were done by the same team of workers, patients being in the recumbent position, with knees flexed; the Hackett notation was used.

As will be seen from the map on page 154, the survey extended from the western to the extreme eastern frontiers of northern Greece, along the Yugoslav, Bulgarian and Turkish borders, as far east as the Maritza river. Villages and towns were selected so as to include examples from both the mountain and the plain regions, in areas where *A. superpictus* or *A. elutus* or both were present.

* Detailed data relating to the individual villages surveyed in the various areas are deposited at the Royal Society of Tropical Medicine and Hygiene and in the Wellcome Trust Research Laboratories, London.

If the figures given in this survey are considered with those published for the spring survey of 1945-46 (Foy, Retter *et al.*, 1946), a fairly comprehensive picture of the malaria situation in northern Greece will be gained.

As stated above, the object of the present survey was to gain information regarding the transmission of malaria during the summer of 1946. It must be admitted, however, that, owing to the complexity of the problem and to the chaos reigning in Greece, this object has only partly been achieved.

One of the striking features which the survey has revealed is the very low blood-indices compared with spleen-indices, and the shift of the spleen-indices to the left. In the past, the ratio between the blood- and spleen-indices has been of the order of 1 : 1.3-1 : 3.5. This ratio is subject to considerable variation from season to season, dependent upon changes in malaria transmission. Further, there will obviously be variations in the ratio in the different age-groups. In Macedonia and Thrace the blood- and spleen-indices tend to approach one another in magnitude when the parasite-index rises, and to diverge from one another when the parasite-index falls. In Greece the spleen-index always exceeds the parasite-index, the average parasite-spleen ratio being about 1 : 1.5 in a 'wet area' (see Barber *et al.*, 1936) in the autumn of 1934, changing to 1 : 1.24 in the autumn of 1935. In another group of villages (dry) (see Barber *et al.*, 1936) where the parasite-index fell, the ratio changed from 1 : 2.84 to 1 : 3.56. In other words, when transmission is high the parasite- and spleen-indices tend to approach one another more nearly; when the transmission is low the divergence between the two indices is wide.

These changing ratios can be used as a measure in assessing the transmission-rate from year to year. As shown by Balfour (1935), the general picture for Greece was a mean spleen-rate of 35.6 per cent. and a mean blood-rate of 17.4 per cent., giving a parasite-spleen ratio of 1 : 2.4. From 1932 to 1938 the blood-indices in general were about half the spleen-rates, with, of course, local differences due to variations in transmission.

The situation in the winter of 1946 was vastly different from this. Of the 11,905 children examined 3,582 had positive spleens, giving a spleen-index of 30 per cent., distributed as follows: P = 68.8 per cent.; I = 25.6 per cent.; II = 4.7 per cent.; III = 0.6 per cent.; IV = 0.1 per cent. The figures for the various regions of Macedonia and Thrace show very wide local differences. Regarding the parasite-rate of the children, out of 7,331 bloods examined there were only 75 positives, giving a parasite-index for the whole area of only 1 per cent. and a parasite-spleen ratio of 1 : 30. The distribution of species among these 75 positives was as follows: *P. falciparum* 42 (56 per cent.), *P. vivax* 31 (41 per cent.), *P. malariae* 2 (3.0 per cent.), gametes 41 (55 per cent.). This ratio of 1 : 30 for the whole region of Macedonia and Thrace is something quite extraordinary, and, taken with the equality in the number of *P. vivax* and *P. falciparum* in the positive slides, appears to indicate a season of extremely low transmission in 1946, with low endemicity in places which in previous years had suffered hyperendemic conditions. The blood and spleen survey carried out in the spring of 1946 (Foy, Retter *et al.*, 1946) revealed a similar state of affairs, with relatively high spleen-rates and very low blood-indices. In certain of the areas there was a marked drop in the spleen- and blood-indices between the spring and winter surveys. In Gida village, for example, the spleen- and blood-rates fell from 70 per cent. and 26 per cent. to 41 per cent. and 1.3 per cent. respectively, 75 per cent. of the spleen-rates falling in the P and I classes. Similarly, in Nea Halkidon, where the spring spleen- and blood-rates were 42 per cent. and 12 per cent. respectively, they fell to

19.0 per cent. and 0.0 per cent. in the winter survey. Mikis, on the other hand, in the Xanthe region, had spring spleen- and blood-rates of 69 per cent. and 37 per cent., falling only to 61 per cent. and 28 per cent. in the winter. All these areas were treated with DDT.

There are, of course, very wide variations in the parasite- and spleen-indices and in their ratios in the different regions, and a more balanced picture of the malaria transmissions during the 1946 season can be obtained from an analysis of various regions. For example, the Salonika region is an urban area containing one or two semi-rural townships. The spleen-rate for the whole of this area was 16.2 per cent., with a parasite-index of 0.48 per cent. and a ratio of 1 : 32. The area has not been a highly malarious one since 1935, and most of the infected cases have either occurred in Triendria or Diavata or been contracted outside the region. Gida, on the other hand, is situated in the centre of a swampy plain, formerly highly malarious, with an abundance of *A. elutus*; the mean spleen-index of this area was 39.8 per cent., with a blood-index of 13.4 per cent. and a ratio of 1 : 3. Like the Salonika area, the Gida region was treated with DDT several times during the 1946 season. It will be seen that the parasite-spleen ratio here was very much nearer unity than in the Salonika area, pointing to a greater volume of transmission in the Gida region. In Negrita, usually regarded as a bad malarious area, the spleen-index was 38.9 per cent., with no positive bloods in the 239 slides examined. In the Serres region the spleen-index was 48.6 per cent., with a blood-index of 0.7 per cent., giving a ratio of 1 : 70. In Mikis, in the Xanthe region, a mountain village, about 1,000 metres up, with innumerable mountain streams, where *A. superpictus* is the sole vector, the spleen-index was 61.2 per cent. and the blood-rate 28 per cent., giving a ratio of 1 : 2.2. A survey of this village in the spring of 1946 showed a spleen-index of 69 per cent. and a blood-rate of 37 per cent., the ratio being 1 : 1.9. It so happens that this village was treated with DDT a number of times during the winter of 1945 and the spring of 1946 on account of a typhus epidemic which had its centre there. This village, with its parasite-spleen ratio of 1 : 1.9 in the spring, changing to 1 : 2.2 in the winter, most nearly approaches the situation that existed in Greece in the 1932-39 period. In the Serres region, Peponia, which was treated with DDT, had a spleen-index of 82.4 per cent. and a blood-index of 4.0 per cent., with a ratio of 1 : 20. Skitholithos, with a spleen-index of 94.4 per cent., had no positive bloods in 36 examined; this area was not treated with DDT.

From these examples it is clear that there are great variations in parasite- and spleen-indices in the different regions, and wide divergences in the parasite-spleen ratios, indicating very wide differences in the volume of transmission in the various areas surveyed. These variations do not appear to be in any way correlated with DDT treatment (see below).

Turning to spleen sizes, it will be seen that the bulk of the enlarged spleens fall into groups P and I, with a much smaller proportion in group II and very few in groups III and IV. This shift of the spleen sizes from the larger categories to the smaller ones indicates diminished transmissions operating over a period of several seasons.

V. THE 1946 SURVEY OF INFANTS UNDER ONE YEAR OF AGE

The doubtful value of spleen-indices in very young children induced us to omit this examination. Out of 2,207 infants examined, varying in age from three months to one year, none had lived through more than one transmission season. The bloods were taken in November-December, and each child had its blood examined once only. Out of the

2,207 bloods taken there were only 21 positives, giving a parasite-index of 0.9 per cent. for the whole area, compared with 1.0 per cent. among the school-children. The parasites found were distributed as follows: *P. falciparum* 8 (38 per cent.), *P. vivax* 12 (57 per cent.), *P. malariae* 1 (5 per cent.); 14 (66 per cent.) had gametocytes. This very low parasite-index, taken in conjunction with the species distribution, indicates a season of very low transmission, while the high percentage showing gametocytes indicates the importance of infants as a reservoir of infection.

In assessing the significance of the parasite-indices of very young children, certain factors must be taken into consideration. Of these, length of exposure to transmission, the age of the infant, and the time at which the blood was taken for examination are of the highest importance. In Greece a very small amount of transmission occurs in May and June, and in October and November transmission, if it occurs at all, is minimal. The maximum rate of transmission occurs in July and August, with September generally relatively unimportant in most years, but becoming more so in years when *A. superpictus* is abundant or in regions where this species is dominant. From these facts it follows that bloods taken during November and December from children born during the preceding July and August will have a higher parasite-index than bloods taken during November and December from children born, say, in September, October and November, although both groups have lived through only one transmission season. Similarly, children born during May and June who have their bloods examined in early July will show a lower parasite-rate than those born in July and August whose bloods are examined in September. An additional important consideration is that, if children born in September, October and November are included with those born in the earlier part of the summer, their number will 'dilute' the parasite-index of the total, since their chances of becoming infected are much less than those of children born in June, July and August. In the present survey none of the infants had lived through more than one transmission season, and all of them had their bloods taken in November and December. But, as they were born in any of the months between May and mid-November, the parasite-index of infants shown in this survey will be almost the lowest obtainable, because of the dilution factor introduced with the children born in the latter part of the season, when transmission is very low. The extent of this dilution will depend on the relative number of children born in September, October and November included in the survey. Another factor of importance is the fact that infants appear to be less liable to infection than older children (Barber *et al.*, 1936), either because of an immunity, which they lose as they get older, or because the older children are out of their swaddling-clothes and are thus more likely to fall a prey to mosquito-bites.

The parasite-index of infants shows wide variations from region to region and from village to village, as was the case with the parasite- and spleen-indices among the school-children. No doubt these variations are dependent upon anopheline density and the presence of gametocyte reservoirs. For example, in the Filippi region the parasite-index of the infants was 8.7 per cent., all the positives being found in one village, Kalamon. In the Serres region, of 17 villages examined only two, Peponia and Skotousa, had parasite-positive infants, and both were villages with high spleen-rates (82 per cent. and 54 per cent.). There appeared to be no constant relation in the villages between high spleen-index and high infant parasite-index.

Similarly, there appeared to be no clear relation between parasite-indices among

infants and the use of DDT. The Filippi region was one of those which were adequately treated with DDT at the proper time, yet the parasite-index of the infants was the highest in the whole of the survey. And in Florina, which was also treated with DDT, the parasite-index of infants was the second highest, at 4.4 per cent. Of the two villages in the Serres region which had parasite-positive infants, one had been treated with DDT and the other had not.

In the Evros region 18 villages were surveyed, 1,528 spleens were palpated, 913 children's bloods were examined and 311 infants' bloods were taken. Of these 18 villages 12 were either not treated with DDT at all or were done at such a time that the work was useless for the vectors concerned. Of these 12 untreated villages, two had parasite-positive infants (16 per cent.). Of the six treated villages, one had a parasite-positive infant (16 per cent.). Similarly, three of the positive bloods of school-children were found in untreated villages (25 per cent.), and only one in the treated villages (16 per cent.).

It should, however, be noted in interpreting these figures that 50 per cent. of the average spleens had a value above 0.80 in the areas not treated with DDT, whilst only 13 per cent. were above 0.80 in the treated areas, indicating that the weight of previous malaria incidence was very considerably heavier in the untreated areas than in those treated. The exact relation of spleen volume—which, after all, is based on linear surface measurements—to the question of immunity is by no means clear. Foy, Retter *et al.* (1946) found that plasma proteins in Greece were on the high side of normal throughout the country, but found no relation between splenic enlargement and high plasma proteins.

The precise interpretation of these parasite-indices of infants is rendered more difficult on account of the absence of any previous survey done by the method used in the present survey. Barber *et al.* (1936) give figures of the infants examined, but they took bloods on several different occasions from the same child, and a positive occurring in any specimen was reckoned in calculating their index; their figures are therefore not comparable to those taken in this survey, where only one blood was taken from each infant. Balfour *et al.* (1935) in their surveys found parasite-indices of infants varying from 4 per cent. to 47 per cent. in bloods taken during October, November and December, 1930–32, the figure of 47 per cent. being for Nea Carvali, a village of extremely evil repute for malaria, with spleen-rates of 90–100 per cent. and parasite-indices of 50 per cent. and 80–100 per cent.

In the Filippi region, an area well treated with DDT, the blood-index of infants was 8.7 per cent.; and in the village of Kalamon in the same region the parasite-rate was 13.3 per cent. among 30 infants examined. The ages of the positives were nine months (*P. vivax*), 12 months (*P. falciparum*), 11 months (*P. falciparum*) and 11 months (*P. vivax*), and of the four positives two had gametes (one *P. falciparum* and one *P. vivax*). It is probable that had the parasite-rate of infants been carried out by any other method than the one used here this rate for the Filippi region and for Kalamon would have been considerably higher.

Livadas and Sphangos (1941) have published parasite-indices of infants for 1934, giving rates of 0.0–50 per cent. In most of their cases only small numbers were examined (two in the case of the 50 per cent. example), and no information is given of how often the bloods were taken or how many months of transmission the children had lived through, or in which month of the year the bloods were taken. Comparison of the figures given by Livadas and Sphangos with those of the present survey are therefore not possible.

In a more recent paper Livadas and Belios (1946) report the results of spleen and para-

site surveys in villages treated with DDT and untreated. No rates for infants were available for any of these areas prior to 1946.

VI. THE 1945-46 DDT CAMPAIGN

The DDT used in the campaign in Macedonia and Thrace was a 5 per cent. solution, in kerosene for the interior of houses and in Diesel oil for outhouses and stables, etc. (about 2.0 gm. per square metre). For spraying by aeroplane a 20 per cent. solution in Velsicol was used, 500 c.cm. per acre. This mixture was also used diluted in Diesel oil for spraying

TABLE V

Examination of the spleen and blood of school-children and infants in DDT-treated and untreated areas

Region	No. of spleens examined	No. of spleens positive	Spleen-index percentage	No. of children's bloods examined	No. of children's bloods positive	Children's parasite-index percentage	No. of infants' bloods examined	No. of infants' bloods positive	Infants' parasite-index percentage
DDT AREAS									
Salonika ...	924	135	14.6	558	2	0.4	149	0	0.0
Langada ...	700	132	18.9	377	1	0.3	151	1	0.7
Gida ...	617	245	39.8	398	11	13.4	82	0	0.0
Katerini ...	379	44	11.6	309	5	1.6	84	3	3.5
Edessa ...	450	57	12.7	280	1	0.4	75	1	1.3
Florina ...	249	39	15.7	210	3	1.4	33	1	3.3
Negrita ...	180	42	28.3	120	0	0.0	39	1	2.6
Xanthe ...	678	209	31.0	491	20	4.0	212	2	0.9
Komotini ...	1,098	329	29.9	728	0	0.0	234	1	0.4
Serres ...	574	335	58.6	274	3	1.1	96	1	1.0
Filippi ...	251	97	38.2	152	7	4.6	46	4	8.7
Kavalla ...	441	150	34.0	266	1	0.4	55	0	0.0
Alexandropolis	373	98	26.3	217	2	0.9	50	0	0.0
Evros ...	450	157	34.9	257	1	0.4	73	1	1.3
Totals ...	7,364	2,069	28.2	4,637	57	1.2	1,379	16	1.2
NON-DDT AREAS									
Salonika ...	115	33	28.8	68	1	1.5	20	1	5.0
Langada ...	183	41	22.4	100	1	1.0	47	0	0.0
Florina ...	184	30	16.3	140	1	0.7	13	1	7.7
Negrita ...	197	104	52.8	119	0	0.0	12	0	0.0
Xanthe ...	227	26	11.5	170	2	1.2	122	0	0.0
Komotini ...	315	53	16.8	100	0	0.0	27	0	0.0
Serres ...	929	301	32.4	471	4	0.8	153	1	0.7
Alexandropolis	447	158	35.1	285	0	0.0	100	0	0.0
Evros ...	1,078	99	8.4	656	5	0.8	238	2	0.8
Totals ...	3,675	845	23.0	2,109	14	0.67	732	5	0.7
	7,364	2,069	28.2	4,637	57	1.2	1,379	16	1.2
Grand totals	11,039	2,914	25.5	6,746	71	1.06	2,111	21	1.0

outhouses. The cost of the campaign for 1946 was estimated at £300,000. In Table V are given the parasite- and spleen-indices among school-children and infants both in areas which had been treated with DDT and in those which had not.

In the treated areas the mean parasite-rate among 4,637 children was 1.2 per cent., and the mean spleen-index among 7,364 children was 28.2 per cent., giving a parasite-spleen ratio of 1:23. The mean parasite-index among 1,379 infants in the treated region was 1.1 per cent.

In the untreated areas the mean parasite-rate among 2,109 school-children was 0.67 per cent., and the mean spleen-index among 3,675 children was 23.0 per cent., giving a parasite-spleen ratio of 1:34. The mean parasite-index among 732 infants was 0.7 per cent.

There is clearly very little difference in the indices between the treated and the untreated areas when the figures are considered in this way. Looked at from the point of view of the number and type of areas showing positives, we find that in 14 treated areas 10 yielded parasites among infants (71.0 per cent.) and that in nine untreated areas four yielded infants with parasites (44.0 per cent.). For school-children, 12 out of the 14 treated areas gave positive bloods (86.0 per cent.), and six out of the nine untreated areas (66.0 per cent.). Further, the mean spleen-rate of 28.2 per cent. in the treated areas was 3.0 per cent. above the mean spleen-index for the two areas combined, whilst the mean spleen-index for the untreated areas was 2.0 per cent. below the mean for the combined areas. Similarly, among school-children the mean parasite-index for the treated and untreated areas was 1.06 per cent.; the mean for the treated areas was slightly above this, at 1.1 per cent., and that for the untreated areas slightly below, at 0.67 per cent.

Looked at from these aspects, it does not appear that there are any striking differences in the indices for the treated and for the untreated areas, though admittedly the grouping together of widely separated regions, all with possible transmission differences, does not give a perfect picture of the situation.

Blood- and spleen-indices in the different villages of the same region, especially when the villages are near together and possibly subject to the same anopheline and other conditions, are more likely to yield useful information; but, as no specific control areas were set aside when the work was started, such valuable data are not forthcoming. However, the Evros region comprises a fairly uniform area, although not without wide differences in malaria incidence, anopheline density and species, which make it far from ideal for splitting into homogeneous sections. In this region, as was pointed out above, 18 villages and townships were surveyed, 1,528 spleens were palpated, and the bloods of 913 school-children and of 311 infants were examined; yet no consistent correlation was found between these indices and treatment with DDT, and any differences which were shown were too small to be of any significance and could be explained on other bases. What perhaps was more significant was the fact that weight of previous malaria, as shown by the average spleen, was considerably higher in the untreated areas than in those treated, and this must be taken into account in assessing the place occupied by DDT in the picture.

From the figures available to date, no precise conclusions can be drawn one way or the other as to the place which DDT occupies in relation to the reduction in malaria incidence in Macedonia and Thrace. There can be no question that there has been a most significant fall in malaria morbidity and mortality during the past five years—a fact clearly shown by changed parasite-spleen ratios, steep falls in malaria incidence, extremely low parasite-indices over all the areas surveyed, and low mortality figures. Transmission-rates appear also to be much lower, although absence of data collected in a similar way to that used in the present survey makes comparisons hazardous. How far these conditions are due to the use of DDT is extremely questionable; in our opinion the insecticide has played a very limited part. As has been pointed out above, the steep fall of the incidence of malaria had started long before DDT was introduced into Greece. The nature of the country and its inaccessibility over large areas both on account of terrain and of turmoil,

as well as the breeding-habits of one of the important vectors, *A. superpictus*, make control, even under the most ideal conditions, a matter of great difficulty. Moreover, the construction of the houses and stables, as well as the habits of the peasants, who often sleep out of doors during the summer months, make adult-control measures none too easy a problem.

There can be no question that DDT is an insecticide of a high order of potency, but its use in a country such as Macedonia or Thrace appears to be a good deal more complex than was originally anticipated. If it is established that anophelines can 'select' the sites on which they roost, the problem becomes even more difficult, and to the simple spraying of walls and ceilings with concentrations of DDT which leave an adequate residue will have to be added perhaps the contents of the rooms, stables, etc. And a further factor which may have to be considered is the light-response of mosquitoes, recently investigated by Kennedy (1947). It is, of course, unnecessary to destroy all the transmitting mosquitoes; it is only necessary to reduce their numbers to a certain minimum level or so to shorten their length of life that even if they take an infected meal they die either before they develop sporozoites or before they have an opportunity to bite a victim.

A further point of importance in Greece which may have to be taken into account is the silk-worm industry, usually located in areas where water is abundant. Indiscriminate DDT-dusting from aeroplanes can cause, and has caused, havoc in certain regions. Since nearly every house in such regions rears silk-worms, house-spraying must be done with the utmost discrimination or not at all; in such areas, perhaps Paris green is the method of choice for the control of larvae.

In conclusion, it should be added that until more data of a critical nature are available, and until work with DDT has been going for a longer period, judgement must be withheld regarding the part which the insecticide has played in the reduction of malaria incidence in Greece during the last six years. But, in view of the fact that it was introduced at a time when the malaria incidence was falling steeply, it would seem that its effect was minimal.

SUMMARY

1. A country-wide survey of Macedonia and Thrace was made during November and December, 1946, to ascertain the effect of DDT on malaria incidence and its relation to the low malaria-rates then prevailing.

2. Nearly 12,000 spleens were palpated, and 7,000 bloods from school-children were examined, together with 2,200 bloods from infants who had lived through only one transmission season.

3. The parasite-spleen ratio had fallen from its previously usual figure of 1 : 2-1 : 4 to the low level of 1 : 30-1 : 70. The spleen-rate for the whole area of Macedonia and Thrace was 30 per cent., with a parasite-index of 1 per cent. among the school-children.

4. The general trend of malaria during the past 25 years in the rural areas of Macedonia and Thrace has been a downward one. In 1942 there was a great malaria epidemic, the incidence trebling itself in that year in both rural and urban areas. Proportionate mortality similarly rose from 4.9 per cent. to the unprecedented figure of 15.9 per cent. The reasons for this great rise in malaria incidence were complex and are discussed.

5. Following this epidemic there was a very sharp fall in both incidence and mortality in 1943, which was continued in 1944, and to a lesser extent in 1945 and 1946. The reasons for this steep fall are analysed and discussed. An analysis of the spleen-, blood- and transmission-indices in DDT-treated and untreated regions revealed that there was no significant differences between the two, and an examination of the 1940-46 data makes it quite evident that the major fall occurred in 1943 and was well established before DDT was introduced into Greece in the latter part of 1945. From the evidence available it would seem very doubtful if DDT contributed much to the malaria reduction in Macedonia and Thrace.

6. The difficulties are discussed of carrying out an antimalaria campaign in a country like Macedonia and Thrace, where *Anopheles superpictus* is an important carrier and where peasant housing-conditions and habits are primitive. If recent work on the habits of mosquitoes in relation to DDT are confirmed, these difficulties will be greatly enhanced.

7. The significance of the fall in incidence of blackwater fever is discussed in the light of malaria immunity and atebriization.

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FRUIT-EATING BATS AND PARALYTIC RABIES IN TRINIDAD

BY

J. L. PAWAN

(From the Government Bacteriological Laboratory, Trinidad)

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It has been shown by Hurst and Pawan (1932) and by Pawan (1936*a*, 1936*b*) that the paralytic rabies of man and animals in Trinidad is spread by the bite of the blood-lapping vampire bat (sp. *Desmodus rotundus murinus*, family Desmodontidae). The natural food of the vampire consists of mammalian blood. In order to obtain its meal of blood it must therefore inflict a wound on an animal or man. In this normal act of biting, an infected vampire may spread rabies to its victims. Fruit-eating bats also abound in Trinidad. They do not require blood for their food, but live on fruits. With them the necessity for biting a mammal to obtain a blood-meal does not exist, and theoretically they may be regarded as no source of danger. But if a docile animal, such as a dog, horse or mongoose, may, when infected with rabies, depart from its normal habit and bite its master, the possibility arises that a rabid fruit-eating bat may bite a mammal, not for the purpose of obtaining blood, but because it is rabid. Fruit-eating bats belong to the family Phyllostomidae, which is readily distinguished by the presence of a well-formed leaf- or spear-like appendage on the nose and by a characteristic dentition. This family is a large one in Trinidad, and, while including fruit-eating bats, does not include the blood-lapping vampire. Among the fruit-eaters, *Artibeus planirostris trinitatis* is the commonest species found locally.

Bats are communal and gregarious mammals, but their gregariousness is an exclusive and select one. In their common roosting-places they nestle side by side, but the various families and species avoid close association. They repel contact with a visitor from an alien family, and link themselves only with members of their own species and family. Homer's inimitable imagery of the souls of the dead huddling in Hades and being driven forward, gibbering, by Hermes (*Odyssey*, xxiv) must bear reference to one species—possibly the fruit-eaters—from his emphasis on their shrill squeaking:

'As bats in hollow of mystic cavern, whenever any of them has dropped out of the string and falls from the rock, fly shrilling, and cling to one another, so did they with shrilling cry hold together as they moved.'*

In their efforts to keep to their own kind, vampires bite and repel fruit-eaters, and, if infected, may therefore transmit rabies to them under natural conditions. Attacks of one species by another have been witnessed in caves and elsewhere in Trinidad.

The bat which Haupt and Rehaag (1921) discovered as the first infected with rabies in Blumenau, Brazil, possessed a definite leaf-like structure on its nose, and therefore did not belong to the blood-sucking Desmodontidae family. It was found in a protected stall

* Quoted by Plato, *Republic*, iii; Jowett's translation.

on an eight-day-old calf, and available evidence suggested that it had bitten that particular calf, which subsequently developed paralytic rabies. But 'leaf-nose' bats do not feed on blood. The possibility therefore existed either that there was an error in the zoological identification or that fruit-eating bats may in abnormal circumstances bite mammals. Haupt (Haupt and Rehaag, 1921), also in Brazil, had previously examined the stomach contents of bats (not the large flesh-eating *Vampyrus*) bearing leaf-like appendages on their noses. In two instances he found partially digested blood, in one the stomach was empty, and in another there was banana. It would seem, therefore, that 'leaf-nose' Phyllostomidae bats, normally non-blood-feeders, may at times ingest blood. When, at a later period, Rehaag (Haupt and Rehaag, 1921) was able to produce paralytic rabies in rabbits and guinea-pigs by the intramuscular injection of the brain of a 'leaf-nose' bat (*Phyllostoma superciliatum* Burmeister, 1854), the evidence incriminating 'leaf-nose' bats as transmitting agents, through their bites, of paralytic rabies in animals in Brazil seemed almost conclusive.

In 1931, during the height of the Trinidad epidemic of paralytic rabies, about 200 bats caught in the city of Port of Spain and in certain rural districts were examined indiscriminately without reference to species, and the first found to be infected with rabies was a 'leaf-nose' bat which had been caught flying during the day in a chemist's shop. At the time of its capture the distinguishing features of fruit-eating bats were known locally, and there was no doubt that this bat was the common fruit-eating *Artibeus planirostris trinitatis*. After the fact had been established that bats were the transmitting agents of the disease, efforts were naturally concentrated on the examination mainly of those found biting or attacking animals or man, and the conclusion was reached that the blood-lapping *Desmodus* was the species concerned. But in view of the experiences recorded from Brazil, and of the fact that a total of five fruit-eating *Artibeus* had been found in Trinidad with Negri bodies in their brains, it became imperative to determine definitely whether fruit-eaters do at times bite mammals and so produce rabies. For this purpose the following experiments were undertaken, by the methods previously described (Pawan, 1936b).

Experiment No. 1

On December 7th, 1937, six *Artibeus* bats were caged with a vampire which had been infected 42 days previously with *virus fixe*. Five died within the following five days, one showing bites on the forearm, chest and abdomen. The sixth, which had been kept with the vampire for seven days, was alive and well up to February 7th, 1938, without any evidence of disease. On that day it was killed and the saliva was scarified into the abdominal wall of a rabbit. No Negri bodies could be seen in its brain. On February 1st the rabbit showed typical paralysis of the left side and died on the same day, but Negri bodies were not seen.

Experiment No. 2

On January 14th, 1938, five *Artibeus* were caged with this same vampire. On February 10th, i.e., 27 days later, three of them were removed and their saliva was scarified into the abdominal wall of a rabbit. On the 21st, i.e., 11 days later, the rabbit showed paralysis of the hind quarters and died on the same day. The brain showed no Negri bodies, but it was injected, also on the same day, intrasciatically into a guinea-pig. The guinea-pig died on March 8th, i.e., 15 days later, and its sciatic nerve was injected into the

sciatic nerve of another guinea-pig. This process of intrasciatic inoculation was continued into four further guinea-pigs in succession, and Negri bodies were seen in the fourth. The *Artibeus* bats remained alive and well, without any abnormal symptoms, for 36, 42, 47, 80 and 95 days respectively.

Experiment No. 3.

On July 28th, 1938, five *Artibeus* were injected with a human strain (V.J.) of rabies virus.

No. 1 died three days later. No. 2 remained well, but was found dead on August 5th. Nos. 3, 4 and 5 showed no sign of disease and were caged with two rabbits on August 18th. No fruit was placed for them, but grass was provided for the rabbit. On the 21st two of the bats were found dead. On the 25th the third died. The rabbit was not bitten. None of the bats showed any abnormality, but Negri bodies were seen in no. 4.

Experiment No. 4

On July 29th, 1938, four *Artibeus* were injected with the same human virus. On the mornings of August 11th, 12th and 13th pieces of wood from the cage were found lying on the floor. On August 29th one bat was found dead and there were numerous Negri bodies in the brain. On November 11th the second died, but Negri bodies were not seen. On December 4th the third died and showed Negri bodies. On August 2nd, 1939, the fourth died and Negri bodies were present.

Experiment No. 5

On December 18th, 1938, six *Artibeus* bats were injected with *virus fixe*, with the following results.

No. 1 died on the following day. No. 2 died on December 26th, without any evidence of disease. No. 3 was seen biting at the woodwork of the cage during the night and day of January 6th, 7th, 28th, 29th and 30th, and splinters of wood were seen on the floor. On January 6th a white rabbit was placed in the cage, but up to February 5th it was not bitten. On that day bat no. 3 died. No. 4 was flying violently and continuously during the day and night of January 5th, 6th and 7th. It was found dead on the 7th. No. 5 remained well until February 5th, when it died. Negri bodies were not seen in the brains of any of these bats.

Experiment No. 6

On December 22nd, 1938, six *Artibeus* were injected with a strain of bat virus isolated from a vampire, with the following results.

No. 1 died on the following morning. No. 2 died five days later. No. 3, on January 2nd, 1939, would fly violently at the slightest noise and dash itself against the wire of the cage, falling to the floor, where it would remain for a while, and then beginning to fly again. On the following day it was quiet, but on the 4th, 5th and 6th it was again 'violent.' From the 7th it remained quiet and normal until April 17th, when it was found dead. Negri bodies were present in the brain. No. 4 showed wounds from bites by its companions around the buttocks, arms and legs, and died on February 8th. Negri bodies could not be seen. No. 5 became 'violent' on April 2nd. It would emit hissing sounds and fly

about violently. On the 5th it was normal and remained so until June 14th, when it was found dead. Negri bodies were present in the brain. No. 6 showed no abnormality and died also on June 14th. Negri bodies were not seen.

Experiment No. 7

On January 5th, 1939, six *Artibeus* bats were infected with another human strain. No. 1 was found dead on the 7th, two days later. No. 2 died on the 9th. On the 11th and 12th, nos. 3 and 4 were seen biting the woodwork of the cage. At the slightest noise they would dash against the wire and snap at it. On the 14th they were both let loose in a bat-proof stall with a bull calf, and fruits were placed for them. The calf was not bitten on the nights of the 14th and 15th, but during the night of the 16th it was bitten on the tip of the lobe of the right ear and on the lower part of the right foreleg. Bleeding was profuse. No fruit was eaten during the night. On the night of the 17th the calf was not bitten, but half a banana was eaten. On the night of the 18th the calf was again bitten on the lower part of the left hip, and no fruit was eaten. No further biting took place, and the bats appeared normal. On the 19th one of the bats—no. 3—was found dead. It was undoubtedly a 'leaf-nose' fruit-eating bat, and the hippocampus showed Negri bodies. On March 3rd the other bat died and Negri bodies were seen. This was undoubtedly also a 'leaf-nose' fruit-eating *Artibeus*. The calf had been removed from the bats on January 22nd and kept in a bat-proof stall. On April 9th it developed paresis of the hind legs, on the 12th it was paralysed in the hind and forelegs and was salivating profusely. On the 13th, i.e., 86 days after the first bite, it was found dead, and Negri bodies were present in the hippocampus. Bats no. 5 and 6 were vicious on January 16th and 17th. They were snapping at each other and biting at the woodwork. One of them showed a wound on the forearm, and blood was on the floor of the cage. No fruit was eaten during these two days. On the 21st one was found dead and Negri bodies were present in the brain. The other was normal, and on February 26th was caged with another calf. They were kept together for seven days, but the calf was never bitten and the bat showed no abnormality. On March 5th it was found dead. The brain showed no Negri bodies.

Experiment No. 8

On March 16th, 1939, five *Artibeus* were inoculated with a human strain of virus (V.J.). On the 18th no. 1 died, on the 16th no. 2 died, on May 21st no. 3 died, on July 16th no. 4 died, and on July 26th no. 5 died. At no time did any of the bats show evidence of disease, and Negri bodies could not be seen in the brain of any.

COMMENTS

1. As with the blood-sucking vampire bat, fruit-eating *Artibeus* bats may prove refractory to infection with rabies virus; but, though they may not manifest any evidence of disease, their saliva may carry infection. Fruit-eating bats, when infected with rabies, may bite mammals, not necessarily with the object of obtaining blood for food, but on account of a change in habit through being rabid. Mammals so bitten may develop rabies. Fortunately, in nature vampires live in communities separate and distinct from fruit-eating bats, and facilities for the spread of an epizootic of rabies from vampires to *Artibeus* are therefore limited.

2. During the height of the 1929-31 epidemic of rabies in man and animals in Trinidad, authentic accounts were given of bats flying from fruit-trees and attacking animals and even man. The probability is that such bats were fruit-eaters.

3. While the destruction of the blood-feeding vampire remains the main objective in antirabies bat campaigns, it should not be forgotten that fruit-eating bats, when rabid, may bite mammals, and measures for reducing their numbers should not be lessened.

4. The average length of life of the *Artibeus* after artificial infection with rabies is 130 days.

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OBSERVATIONS ON A STRAIN OF *LEISHMANIA TROPICA* AFTER PROLONGED CULTIVATION: NOTES ON INFECTIVITY AND IMMUNITY

BY

S. ADLER

AND

AVIVAH ZUCKERMAN *

(From the Department of Parasitology, Hebrew University, Jerusalem)
(Received for publication March 23rd, 1948)

The observations recorded in this paper were made on a strain of *Leishmania tropica* originating from a wild sandfly, passed through man and subsequently maintained in culture for a period of 22 years.

The history of the strain is as follows. A female *Phlebotomus papatasi* was caught in Jericho on October 26th, 1925, and was dissected in Jerusalem on November 2nd. Between these two dates the insect had no opportunity of feeding. On dissection a heavy infection of leptomonas was found extending from the proboscis to the rectum. The upper part of the cardia was completely choked by flagellates, large numbers being attached to the oesophageal valve and epithelium. The oesophageal diverticulum was found contracting on an almost solid mass of flagellates immediately behind its junction with the oesophagus.

Material from this sandfly was inoculated into two scarified points on the left forearm of a volunteer. On November 27th the patient noticed two papules on the site of the inoculated points, his attention being drawn to them on account of itching. He was examined on November 29th and numerous Leishman-Donovan bodies were found in smears from the papules. Subsequently the lesions developed into typical oriental sores.†

The strain obtained from this case, and labelled 'Ber,' has since been maintained continuously in culture on Locke-serum and Locke-blood-agar. During the last 22 years it has undergone about 800 passages on these media.

Two years after its isolation the behaviour of the strain in sandflies, *P. papatasi*, was examined by feeding the insects through a membrane on suspensions of flagellates of known concentration, and it was found to be typical of local strains of *L. tropica*, i.e., after the third day the flagellates had ascended to the neighbourhood of the oesophageal valve. It was also found (Adler and Theodor, 1926) that the strain was indistinguishable from other Palestinian strains by serological methods.

PRESENT INVESTIGATIONS

In 1938 we observed that an Indian strain of *L. tropica*, sent to us by Colonel J. A. Sinton, produced a generalized visceral infection in the Syrian hamster *Cricetus auratus*. Subsequently it was found (Adler and Ashbel, 1940) that recently isolated Palestinian strains also produced visceral infections in these animals after intrasplenic or intraperi-

* Working with a grant from Messrs. May and Baker Limited.

† These data are extracted from a paper by Adler and Theodor (1926).

toneal injection. As the Ber strain is the oldest in our collection, we thought that it would be interesting to test its infectivity for Syrian hamsters. Consequently between August, 1945, and November, 1947, 18 hamsters were inoculated with this strain: of these, nine received a single intrasplenic injection, one received three intraperitoneal injections, two received one intrasplenic and three intraperitoneal injections, and six received one intrasplenic and six intraperitoneal injections. Each intrasplenic injection consisted of 0.2 c.cm. culture on Locke-serum-agar, corresponding approximately to 12,000,000 active flagellates, and each intraperitoneal injection corresponded to about 30,000,000 or more active flagellates. The animals were examined at intervals of 52-367 days; small pieces of spleen were excised and smears were examined, and in nine animals material from the spleen was inoculated on Locke-serum or Locke-blood-agar. In all cases the results were negative.

Controls. Eighteen animals were inoculated intrasplenically with cultures of relatively recently isolated strains (up to two years), and all of them developed a visceral infection, as judged by direct microscopical examination of the spleen between 56 and 142 days after the inoculation. In most animals the infection was heavy and involved the spleen, liver, lungs, lymphatic glands, bone-marrow and dermis. The infection of the dermis resembled that produced in the hamster by *L. infantum* and *L. donovani*, in that it was uniform and was not accompanied by ulceration. In one animal a very heavy infection of the mucosa of the large intestine was found, but the small intestine was not affected.

REINOCULATION WITH INFECTIVE STRAINS

Ten animals which were refractory to the Ber strain were inoculated with strains proved infective for normal hamsters. The challenging inoculation—intraperitoneal in one animal and intrasplenic in the other nine—was administered one week after the last injection and two months after the first injection of the Ber strain. Nine of the animals became infected, and only one, which died 71 days after the challenging dose, was found negative on microscopical examination. The course of the infection in the nine positive animals was not influenced by the previous inoculations of non-infective flagellates; in fact, in six of the animals the infection was heavier than in four controls inoculated simultaneously from identical cultures. Obviously, repeated injections of the living non-infective strain conferred no appreciable immunity against infective strains of *L. tropica*.

EXPERIMENTS ON MAN

After it had been reasonably established that the Ber strain was non-infective for hamsters, we tested its infectivity for man. Recently isolated strains of *L. tropica* are fatal to Syrian hamsters but produce only a relatively harmless lesion in man. We therefore expected a negative result in human experiments with a strain non-infective for hamsters, and were rather surprised at the following result.

On November 22nd, 1946, two volunteers each received 6×10^6 circ. living flagellates intradermally. On July 17th, 1947, one case had a papule about 2 mm. in diameter and the other a minute subcutaneous nodule at the site of the inoculation. The lesion in the second case was so insignificant that it would have been overlooked but for close and continual observation of the site of inoculation. Numerous L.D. bodies were found in smears from both lesions. Cultures were positive. Both lesions grew slowly, and by the beginning of 1948 the first was about 1 cm. and the second about 0.5 cm. in diameter. Cultures isolated from both cases were non-infective for hamsters.

To test the leishmanin reaction, on August 4th, 1947, 0.1 c.cm. of a suspension of flagellates (Ber strain) 1×10^6 per c.cm. in 0.5 per cent. phenol was inoculated intradermally into both volunteers. As a control 0.1 c.cm. of 0.5 per cent. solution of phenol was inoculated simultaneously into other sites. One hour later there was a diffuse reddening on the site of the inoculation of the dead flagellates, which disappeared after two hours. On the following day an area of erythema about 0.5 cm. in diameter appeared on the same site. In the case with the subcutaneous nodule the erythema persisted for seven days and then faded progressively. In the case with the papule the erythema persisted for three days and then faded progressively, until by August 14th it was very faint. On August 24th there was a complete reactivation of the specific reaction: an area of erythema 7.5 mm. in diameter appeared and was still visible on September 10th. On the day following this reactivation two biopsies were performed, one on the site of the lesion and one on the site of the injection of dead flagellates. L.D. bodies were swarming in the lesion, and numerous polymorphs and macrophages (but no parasites) were found in the other site. Cultures from the reactivated site were negative.

Suspensions of phenol-killed flagellates of the Ber strain produced the usual leishmanin reaction in active and cured cases of oriental sore.

The following experiments, carried out on an old case with the following history, are of interest. On June 26th, 1925, flagellates from a naturally infected sandfly caught in Jericho were inoculated into two points on the left forearm of a volunteer. On July 31st L.D. bodies were found in a papule on one of the sites of inoculation. This lesion ultimately developed and reached dimensions of 2×3 cm. Spontaneous cure occurred after about two years from the appearance of the papule.* Frequent attempts to reinfect this case all gave negative results. The skin test was repeated several times between 1927 and 1938 and was constantly positive.

On November 19th, 1946, 0.1 c.cm. suspension in 0.5 per cent. phenol (1×10^6 flagellates per c.cm., Ber strain) was inoculated intradermally into the right forearm, and 0.1 c.cm. of 0.5 per cent. solution phenol was inoculated as a control. After 24 hours an erythematous area 1.5 in. in diameter appeared round the site of inoculation of the dead flagellates. After 48 hours the area of erythema extended to about 4 in. in diameter. After 72 hours it began to recede.

On November 24th another skin test was performed on this case, this time with living flagellates of a strain proved infective for man and hamsters. 0.1 c.cm. of living culture (about 6×10^6 flagellates) was inoculated into a point on the left forearm. This was followed six hours later by a rigor. After 24 hours there was an area of erythema and oedema about 4 in. in diameter containing a central necrotic area 1 in. in diameter. After 24 hours no parasites were found in a smear from the centre of the necrotic patch, but cultures were positive for leishmania and were bacteriologically sterile. The temperature rose to 37.5°C . and on the following day reached 38.5°C .; cultures were negative for leishmania and for bacteria. On November 27th lymphangitis extended over the whole extensor surface of the forearm. The central necrotic area was surrounded by large blisters. Cultures were again negative for bacteria and for leishmania. The patient was confined to bed for three days, and the lymphangitis persisted for one week. By December 1st the area of erythema had receded and the blistered area had become haemorrhagic.

* These details are extracted from a paper by Adler and Theodor (1925).

On the 8th the central area of necrosis was covered by a crust about 1 in. in diameter. On January 15th and 20th, 1947, cultures and smears from the periphery of the necrotic area were negative. The lesion eventually healed, leaving a scarred area about 0.5 in. in diameter.

This is the first record of a typical Arthus reaction induced by a protozoon.

It is interesting to note that in a human case effectively immunized by a previous attack, and repeatedly found to be refractory to reinfection, parasites in small numbers survived for 24 hours and then disappeared. This case is very sensitive to antigens of *L. tropica*, as is shown by the following additional experiment performed after a further interval of nine months. On August 4th, 1947, 0.1 c.cm. suspension of flagellates (Ber strain) in 0.5 per cent. phenol (1×10^6 flagellates per c.cm.) was inoculated intradermally; 0.1 c.cm. of 0.5 per cent. phenol was inoculated into another point as a control. After 24 hours an inflamed area 1×3 in. appeared round the point of inoculation of dead flagellates. After 48 hours the area of reaction grew to 3×4 in. The reaction began to recede on the sixth day. The control was negative.

BEHAVIOUR OF THE BER STRAIN IN SANDFLIES, *P. papatasi*

As previously stated, the Ber strain, when isolated, behaved like a typical local *L. tropica* in sandflies, *P. papatasi*, in that relatively few flagellates produced a high infection-rate and after three days the flagellates were lodged in the vicinity of the oesophageal valve, even when the infections in the insect were slight.

Between August 15th and September 8th, 1947, sandflies were fed through a membrane on suspensions of flagellates of the Ber strain, with the results shown in the following table.

No. of flagellates per 0.1 c.cm. suspension	No. of sandflies dissected after 3 days or more	Results
300	24	3 positive; in 2, flagellates confined to stomach; in 1, flagellates in anterior part of cardia
1,000	20	8 positive; in 7, flagellates confined to stomach; in 1, anterior part of cardia infected
2,000	6	6 positive; in 2, anterior part of cardia infected; in 4, flagellates confined to stomach

The infection-rate in *P. papatasi* fed on suspensions of 300 flagellates per 0.1 c.cm. in the case of recently isolated local strains is usually between 80 and 100 per cent. With the Ber strain the infection-rate is now actually lower than that found by Adler and Theodor (1939) in *L. chagasi* with a similar concentration of flagellates (24 per cent.). The restriction of the flagellates to the stomach in most of the sandflies infected with the Ber strain is even more striking than the low infection-rate.

These observations indicate that the strain is no longer transmissible by the bite of *P. papatasi*. This conclusion is supported by feeding-experiments on a volunteer (no. 1): between August 14th and 26th, 1947, we fed 41 laboratory-bred sandflies, *P. papatasi*, on the lesion of this volunteer; during the same period smears made from the lesion were swarming with L.D. bodies. Only one sandfly became infected; it was dissected seven days after the infecting feed, and flagellates were found in the cardia and stomach.

DISCUSSION

The findings reported above present several points of interest. In the first place they show that the Ber strain originally isolated from a sandfly had changed during years of cultivation: its infectivity for *P. papatasi* had diminished, and its type of behaviour had changed. It is not known whether the strain was originally infective for *Cricetus auratus*, as this hamster was never used as a laboratory animal until 1930 (Adler and Theodor, 1931), but all recently isolated Palestinian strains have been found infective for this animal. In any case, a loss of infectivity for laboratory animals after this long period of cultivation would not be surprising. Parrot (1929) has recorded loss of infectivity of *L. tropica* for mice in one strain after cultivation for one year and 11 months, and in another strain after one year and nine months; a third strain after cultivation for 10 months was infective for mice following intratesticular injection, but not after intraperitoneal injection.*

Our strain was still infective for man and produced oriental sores in two volunteers after an incubation-period of eight months, which is considerably longer than that found in most experimental laboratory infections. In five cases infected with another strain in the laboratory by the bites of sandflies the incubation-period varied from four to eight weeks (Adler and Ber, 1941).

Both volunteers infected with the Ber strain gave a positive leishmanin reaction in the initial stages of the disease, when the lesions were so insignificant that they would have been overlooked but for close and continual observation of the sites of inoculation. The lesions grew and parasites multiplied for a considerable period after the positive reaction had been established. The leishmanin reaction is positive in nearly all active and cured cases of oriental sore, and, though evoked by an antigen present in *L. tropica*, is no indication of immunity against the parasites, for it is present long before the latter have ceased to multiply and before lesions have reached their maximum development. Dostrovsky and Sagher (1946) found the leishmanin reaction positive as early as two days after the inoculation of 2,000,000 flagellates into two volunteers. These authors considered the reaction as an expression of specific sensitivity, and succeeded in transferring it passively to normal human beings by injection of serum from patients with a positive reaction. We found marked sensitivity to both dead and living flagellates in a case which had been repeatedly found refractory to reinfection after spontaneous cure. In this case a very distinct Arthus phenomenon was produced by the inoculation of 6,000,000 flagellates. This is an example of a solid immunity against the causative organism accompanied by extreme sensitivity to a specific antigen; the two processes are not necessarily associated, for, as previously stated, specific sensitivity can exist in the absence of immunity. On the other hand, immunity in the absence of sensitivity has not yet been demonstrated in *L. tropica*. Immunity possibly involves a response to a wider range of antigens than that concerned in sensitivity.

Syrian hamsters did not become immune after repeated injection of living non-infective strains. Parrot (1929) recorded a similar finding in mice inoculated with non-infective strains: out of 12 mice thus treated 10 became infected after intratesticular injection of

* We observed a strain of *L. donovani* isolated in Assam in 1927. In 1930 it was fatal for Syrian hamsters within nine months and produced intense fatty degeneration of the liver. By 1934 infected animals were in good condition after 12 months, and there were no fatty changes in the liver in heavily infected animals.

infective strains and two failed to react. Up to the present, no effective method of immunization against *L. tropica*, apart from the production of an active lesion, has been devised.

SUMMARY

A strain of *Leishmania tropica* obtained originally from a wild sandfly has been maintained in culture for 22 years.

After this period the strain was still infective for man, and two human beings were infected by inoculation of living flagellates.

The incubation-period was eight months in both cases.

The leishmanin reaction was positive during the earliest stages of the infection.

The strain showed a reduced infectivity for sandflies, *Phlebotomus papatasi*. In the majority of infected sandflies the flagellates were confined to the stomach, while in recently isolated strains they ultimately progress to the oesophageal valve and beyond.

Syrian hamsters were refractory to the strain. After passage through man it was still non-infective for hamsters.

Repeated injection, both intrasplenic and intraperitoneal, of living flagellates did not immunize hamsters against infective strains.

An Arthus phenomenon was produced in an immune human being by the inoculation of living flagellates.

Some parasites persisted for 24 hours in the immune case and then disappeared.

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A NEW *AÈDES* FROM THE CAMEROONS,
A. (AÈDIMORPHUS) BONETI S.SP. *KUMBAE* S.SP. NOV.

BY

LEONARD JAN CHWATT

(From the Malaria Unit, Medical Department, Nigeria)

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During a malaria survey made in September, 1947, of some localities in the British Cameroons, a number of mosquito larvae were collected from a rock-pool in the Mambonjeze stream, near to the town of Kumba. The stream flows in a narrow rocky bed at the bottom of a steep wooded ravine, and is densely shaded by a luxuriant vegetation composed of shrubs (*Gaertnera*, *Clerodendron* and *Macaranga*), herbs (*Costus Englerianus* and several Araceae) and ferns (*Marrattia*, *Dryopteris* and numerous Salaginellae).

The following description of a new subspecies of *Aedes* is made from 10 fourth instar larvae, three pupal pelts and two male adults bred out in the laboratory.

Aedes (Aedimorphus) boneti s.sp. *kumbae* s.sp. nov.

LARVA. Length 6-8 mm. Colour dark brown to blackish. Head somewhat broader than long, uniformly pigmented. Antennae slender, slightly more than $\frac{2}{5}$ the length of the head, spiculate, uniformly coloured, except for a narrow dark ring at the base. One long subterminal seta about twice the length of the terminal seta, and one short subterminal seta about half the length of the latter. Antennal tuft at $\frac{2}{5}$ the length of the shaft, with 3-4 simple branches, the longest of which is about half the length of the antenna. Clypeal spines slender, about one quarter the length of the head. Seta 'A' with 6-9 delicate plumose branches; seta 'B' slightly longer than either 'A' or 'C,' with 4-6 delicately plumose branches; 'C' with 7-9 almost simple branches; 'd' very small, situated on the inner side of the base of 'B' with 3-5 simple branches. Mentum in the form of an obtuse-angled triangle, with 13-16 teeth on either side of the large central tooth, the four basal teeth larger than the subterminal teeth and widely separated. Thorax with the spines at the bases of the metathoracic pleural tufts small and blunt, those at the bases of the mesothoracic tufts larger. Abdomen (fig. 1) with the comb forming a triangular patch of about 60-80 delicately fringed scales. Siphonal seta with three simple branches, subsiphonal seta with five plumose branches, anal seta with three stiff simple branches. Siphon dark, with a scale-like arrangement of chitin up to about $\frac{1}{8}$ of the distance from the base to the apex, where the surface becomes gradually smooth and slightly paler. Siphonal index about 3. Subventral tuft at about $\frac{2}{5}$ the distance from the base to the apex of the siphon, with 6-10 plumose branches, approximately equal in length to the diameter of the siphon. A double, not very regular, and rather asymmetrical row of long, strong, dark, simple (occasionally bifid) spines, 6-8 on either side, extending from below the subventral tuft almost to the apex, where they form an irregular bunch of 8-10 spines (fig. 2). The terminal spines are stronger, darker, and almost twice the size of the subterminal spines.

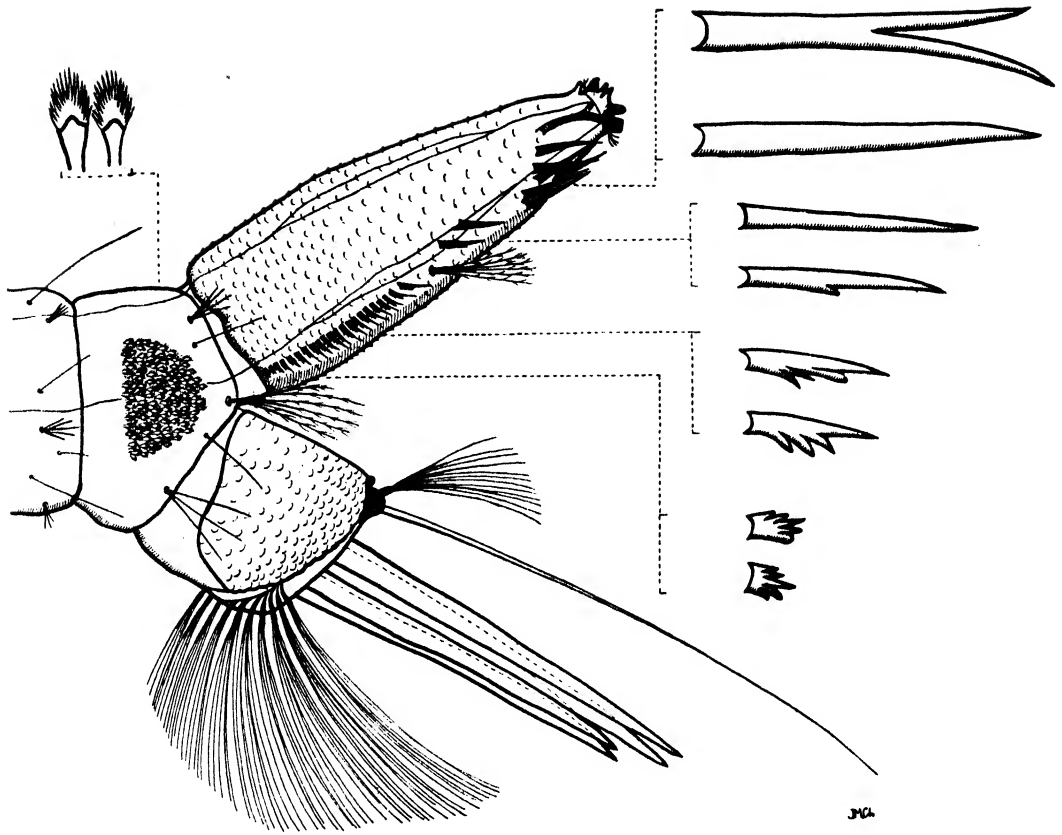


FIG. 1. *A. (Aedimorphus) boneti* s.sp. *kumbae*. Terminal segments of larva.

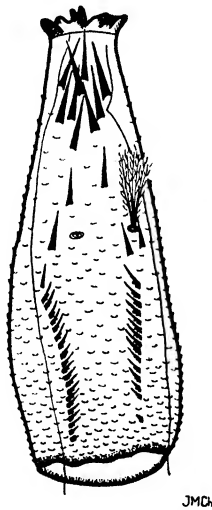


FIG. 2. *A. (Aedimorphus) boneti* s.sp. *kumbae*. Ventral side of siphon of larva.

Pecten composed of 17–22 spines, reaching nearly to the subventral tuft. Basal pecten spines minute, occasionally widely separated from the remainder, with both dorsal and ventral denticles. Typical middle spines with three ventral denticles. One or more of the distal spines have one minute denticle or are simple. The distribution and the number of pecten spines on each side of the pecten are often unequal. Anal segment with the saddle well developed, its chitin scale-like, as in the siphon. Distal edge more heavily chitinized and almost simple. Lateral seta simple. Upper caudal seta with 12–14 branches, lower caudal seta long and simple. Ventral brush with 5–6 paired tufts in the barred area and 2–4 unpaired tufts. Anal papillae lanceolate, the dorsal pair about three times the length of the saddle, the ventral pair slightly shorter.

PUPA. *Integument* of the abdomen with scale-like chitinization. Abdominal segments, particularly the first two, infuscated on the anterior border; last two segments with two dark lateral patches. Genital sacs infuscated. Cephalothorax infuscated on the dorsal surface. *Trumpets* dark, with prominent scale-like arrangement of small pointed chitin-

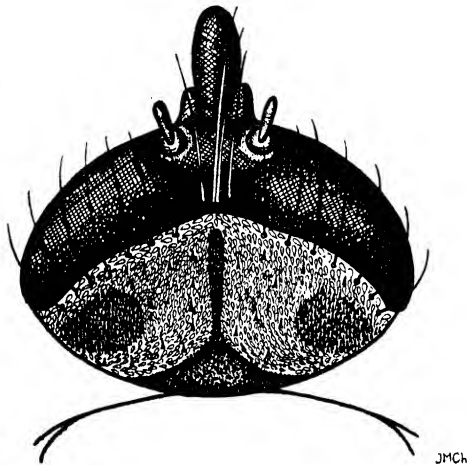


FIG. 3. *A. (Aedimorphus) boneti* s.sp. *kumbae*. Head of male.

plaques. Meatus narrow, cylindrical, $\frac{2}{3}$ of the total length of the trumpet. *Paddles* rounded, symmetrical, with the outer edges toothed and a thickened outer edge along $\frac{2}{3}$ from the base. Terminal bristle about a quarter the length of the paddle, bifid at the tip. *Float hairs* large and dendritic. *First abdominal segment* with seta 'H' short and simple; 'K' long, dark, fairly stout, delicately frayed; 'L' and 'M' with about seven branches; 'S' long and simple; 'T' missing; 'U' very fine and simple. *Seta 'A'* on II–V minute or absent, on VI minute, on VII with 2–3 branches, on VIII with four stout plumose branches. *Seta 'B'* on II–IV simple, slightly longer than the succeeding segments, on V about $\frac{3}{4}$ the length of the segment, on VI and VII about half the length of the segment, bifid at tip. *Seta 'C'* on II well developed, dendritic, on III–VII small, simple to trifid.

ADULT MALE. *Head* (fig. 3) clothed with a mixture of dark upright and narrow pale decumbent scales. Occiput with two, dark, dorsolateral, conspicuous, oval or comma-shaped spots, formed by dark-brown upright forked scales. The remainder of the upright scales pale. Prominent dark-brown bare vertical area along the median suture. Vertical

bristles long and white. Orbital bristles dark. Tori with a few narrow pale scales. Proboscis dark, with a pale labella. Palps about $4/5$ the length of the proboscis, with a pale ring at the constriction of the basal segment; second segment with a few pale scales at the base and at the apex; terminal segment dark, about half the length of the second segment, both with tufts of dark hairs. *Mesonotum* covered with a mixture of narrow, curved, dark-brown and pale scales, the latter broader on the anterior and anterolateral borders and broad and flat around the prescutellar bare area. *Scutellum* clothed with broad, flat, silvery scales. *Pleurae* with patches of pale scales on the prealar, postspiracular and subspiracular areas and on the sternopleura and mesoepimeron. *Bristles* 4-6 pale postspiracular, one row of 6-8 pale sternopleural, no lower mesoepimeral, upper mesoepimeral short and pale. Scutellar and supra-alar long and dark. *Abdominal tergites* II-VI with basal rows of flat grey scales and with patches of pale scales at upper lateral angles of each segment; tergite VII with a median patch of pale scales. Sternites covered almost entirely

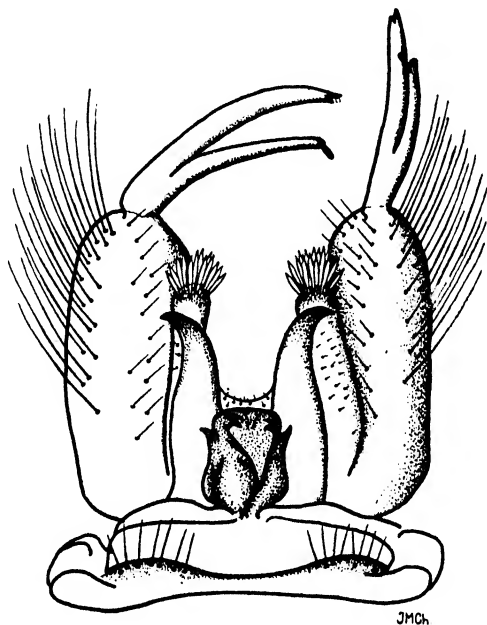


FIG. 4. *A. (Aedimorphus) boneti* s.sp. *kumbae*. Male terminalia.

with broad flat pale scales. *Legs*. Fore femora dark, with a paler basal ventral surface and a few white scales at the distal end of the dorsal surface; middle femora similar, with a rather more extensive pale ventral area; hind femora dark on the dorsal side, except for a white distal spot, mainly pale on the ventral side, with a few aberrant white scales. Fore and middle tibiae dark, with a pale apical spot, about $1/10$ of the tibial length. Fore and mid-tarsi dark; hind tarsi: first segment dark, with an apical white band, second with one basal and one apical white band, the latter about twice the length of the former, third with one apical and one basal narrow band. The fourth tarsus shows some variation: in one specimen it has a dark median band, while in the other the dark scales form only a narrow longitudinal line on the apical half of the lower surface. In both specimens

the fifth tarsus is white but has a similar dark line on the lower surface. *Wings* with scales dark and a small basal patch of pale scales on the lower and anterior surface of the costa. *Terminalia* (fig. 4). Coxite slender, round, with a tuft of long dark hairs on the upper lateral surface and several rows of short hairs on the median surface. Style forked at about $\frac{1}{3}$ of the distance from the base to the tip, split into two long tapering prongs, the lower one bearing a blunt dark terminal spine, the upper one with a short sharp spine near the tip. Basal lobe small, cupuliform, chitinized at the apex, and bearing a bunch of flattened bristles, some of which are stouter and more conspicuous than the remainder. Phallosome tulip-shaped, the outer surface with a prominent tooth on each side near the apical edge, the inner surface with a small row of teeth on each side in a similar position. Paraprocts curved, beaklike, heavily chitinized at the tips. The lobes of the ninth tergite narrow, slightly curved, with seven bristles on one side and eight on the other.

DISCUSSION

In Hopkins's (1936) key to the Ethiopian species of *Aedes*, amended in 1945 by Lewis, the larva described above would run down to *A. (Aëdimorphus) lamborni* Edw., which is obviously incorrect. It differs from *A. lamborni* in the smaller number of branches in the head setae, in the smaller number of comb-scales and of pecten spines, and especially in the presence of the very remarkable spines on the siphon. These spines resemble those described in *Aedes pseudotarsalis* by Someren (1946), but they are more numerous and are distributed throughout the upper part of the siphon, with less tendency to apical bunching. The larva differs from *pseudotarsalis* also in the characters of the pleural spines and of the anal seta, in the more numerous comb-scales, and in the position of the subventral tuft.

In Edwards's (1941) key to the Ethiopian species of *Aedes* the two male adults would run down to *A. boneti* Gil Collado, described in 1936 from a single damaged male captured on the Island of Fernando Po. The similarity between the two adults described above and the original description of *A. boneti* is considerable. Nevertheless, there are several notable differences—mainly the more extensive, rather differently shaped, dark scaling of the head, the pale (instead of golden) colour of the investiture of the mesonotum, the presence of prescutellar rows of scales, the scaling of the abdominal sternites, the presence of small pale apical spots on the dorsal surface of the femora, the presence of basal white spots on the costa, and the markings of the last hind tarsal segments. Comparison of the male terminalia is not possible, as the terminalia of *A. boneti* have not been described. The terminalia of the two males described above are very similar to those of *lamborni*, except in the structure of the phallosome, the much more hairy coxite and the less prominent lobes of the ninth tergite.

This *Aëdimorphus* is described here under the provisional name of *A. boneti* s.sp. *kumbae*. Should the still unknown larva of *A. boneti* prove to be different from the one described above, *A. boneti* s.sp. *kumbae* will have to be treated as a new species, *A. (Aëdimorphus) kumbae*.

The types and cotypes of larvae, pupae and male adults have been deposited at the British Museum (Natural History), London.

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STUDIES ON SYNTHETIC ANTIMALARIAL DRUGS

XX.—THE BLOOD CONCENTRATIONS AND PHYSIOLOGICAL DISTRIBUTION OF SOME HOMOLOGUES OF PALUDRINE IN RELATION TO THEIR ANTIMALARIAL ACTIVITIES

BY

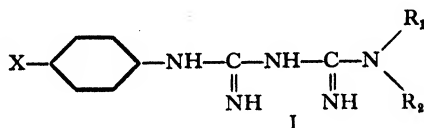
A. SPINKS

(From the Research Laboratories, Imperial Chemical Industries Limited,
Hexagon House, Manchester)

(Received for publication April 2nd, 1948)

INTRODUCTION

Among the new antimalarial agents recently described by Curd, Davey and Rose (1945*a*, 1945*b*) the most active, in avian malaria, were arylbiguanides of general formula I. Of these, 4430 (I: X = Cl; R₁ = CH₃; R₂ = iso-C₃H₇) and paludrine (I: X = Cl;



R₁ = H; R₂ = iso-C₃H₇) have been tested in human malaria. Both were highly effective (Adams, Townshend and King, 1945; Adams, Maegraith *et al.*, 1945; Maegraith *et al.*, 1946; Fairley, 1946). A very large number of related compounds have been tested against *Plasmodium gallinaceum* in the chick (Davey, 1946; Curd and Rose, 1946), and much information is now available regarding the effect on activity of small changes in molecular structure. Among homologues of paludrine (I: X = Cl) very wide variation in activity was displayed, examples of which are shown in Table I. Others have been examined, but these illustrate the most important finding—that compounds as closely related to paludrine as 5093 and 4134 may be completely inactive at high doses.

As yet little is known of the mode of action of antimalarial drugs, but it is certain that a large number of complex factors must be involved. These may perhaps be crudely

TABLE I

Structures of some homologues of paludrine, and their activities against blood forms of *P. gallinaceum* in the chick

Compound	X	R ₁	R ₂	Dose (mgm./kgm.)	Activity
5093	Cl	H	CH ₃	80	—
4134	Cl	CH ₃	CH ₃	80	—
4967	Cl	H	C ₃ H ₇	20	++
Paludrine ...	Cl	H	iso-C ₃ H ₇	10	+++
4430	Cl	CH ₃	iso-C ₃ H ₇	16	+++
3936	Cl	C ₂ H ₅	C ₃ H ₇	80	++
4565	Cl	H	<i>n</i> -C ₄ H ₉	80	++
4635	Cl	H	<i>n</i> -C ₅ H ₁₁	80	++

— = Inactive; ++ = Markedly active; +++ = Highly active (data of Davey, 1946).

divided into two major groups, one governing the access of the drug to the parasite, the other its intrinsic activity against the parasite. The first group, with which this paper is mainly concerned, must embrace the absorption of the drug, its physiological distribution, its rate of removal from the body by degradation and excretion, and its affinity for the parasite. If the intrinsic activity is not known, the favourable nature of these properties can only be assessed by comparing a given compound with one closely related that shows high therapeutic activity. In the present work the blood, plasma and tissue concentrations of a number of homologues of paludrine have been compared with those of paludrine itself under standard conditions. The blood and plasma concentrations give an estimate of the extent by which rate of absorption exceeds rate of removal from the body; the tissue distribution gives an estimate of the selective affinity of the compound for cellular structures. The animals chosen for study were rat, rabbit and chick. The results in the chick obviously have a direct bearing on the activity against *P. gallinaceum*. The results in the rat were obtained before the great difference between the behaviour of paludrine in rat and in chick had been discovered (Butler *et al.*, 1947), but are nevertheless similar to those in the chick. It was hoped that, were an inactive compound poorly absorbed in the chick and well absorbed in the rat, support might be provided for its trial in monkey or human malaria. No compound, however, behaved in this manner.

EXPERIMENTAL SECTION

Analytical techniques. Each compound was determined by the hydrolytic method already described (Spinks and Tottey, 1946a), modified only as dictated by the rate of hydrolysis of a particular compound in N/4 hydrochloric acid. Trial determinations were carried out on water, blood, plasma, liver, lung, spleen, kidney, fat and brain containing known amounts of each compound; and where the recoveries were low the recorded data have been adjusted by means of multiplication factors. Two general trends were observed. The percentage recovery tended to increase with increase in molecular weight, possibly because of a decreased water/benzene partition coefficient; and the recoveries from fat and blood were usually lower than those from the other materials. The lowest recovery observed—of 5093 from blood—was 70 per cent. of the theoretical.

Experimental animals. The experimental animals were albino rats of 180–250 gm., rabbits of 1.5–2.5 kgm., and chicks of 40–60 gm. weight. Either sex was used at random.

Manner of administration. Each compound was administered as an aqueous solution of the hydrochloride. As strong a solution as could be prepared (usually 1–2 per cent.) was used for dosing rats orally and rabbits intravenously. Chicks received 0.20 per cent. solutions. All doses and concentrations refer to the anhydrous base.

BLOOD, PLASMA AND TISSUE CONCENTRATIONS IN THE RAT

Groups of four rats received 80 mgm. of a given compound/kgm., administered by stomach-tube. A group was sacrificed at a desired interval after dosing by withdrawing the maximal volume of heart blood under heavy chloroform anaesthesia. A portion of the pooled, oxalated sample was set aside for analysis; the remainder was immediately centrifuged at 2,000 revolutions per minute for 30 minutes, and the plasma was withdrawn without disturbing the buffy coat. In some experiments selected tissues were also removed and pooled for analysis.

The concentrations of each compound in blood and plasma are shown in Table II,

TABLE II

Blood and plasma concentrations of some homologues of paludrine in the rat after oral doses of 80 mgm./kgm.

Compound	Tissue	Concentrations in mgm./l. after								
		$\frac{1}{2}$ hr.	1 hr.	$1\frac{1}{2}$ hr.	$1\frac{3}{4}$ hr.	$2\frac{1}{2}$ hr.	$3\frac{1}{2}$ hr.	5 hr.	7 hr.	24 hr.
5093 ...	Blood	2.79	2.91	3.60		4.36	4.20	3.94	2.93	2.21
	Plasma	0.79	0.95	1.11		1.25	1.91	0.96	0.95	0.62
4134 ...	Blood	1.44	1.90	1.78		2.20	1.86	1.23	1.41	0.86
	Plasma	0.82	1.02	0.80		1.42	1.52	0.62	0.74	0.52
Paludrine	Blood	1.20		1.58	1.98	1.26			0.70	0.58
	Plasma	0.29		0.73	0.76	0.59	0.47		0.24	0.12
4430 ...	Blood	1.52		0.77		0.53	0.52		0.38	0.15*
	Plasma	0.18		0.46		0.23	0.28	0.11	0.12	0.07*
3936 ...	Blood	1.22	1.06	1.49		0.82	0.77	0.62		0.61†
	Plasma	0.30	0.50	0.30		0.25	0.18	0.25		0.18†
4565 ...	Blood	0.36	0.76	0.66		0.53	0.42	0.39		0.37†
	Plasma	0.13	0.17	0.19		0.14	0.12	0.10		0.12†

* After 30 hours. † After 16 hours.

and those of 5093 in tissue in Table III. The corresponding concentrations of paludrine and 4430 have been previously reported (Spinks, 1946; 1947a); only those in blood and plasma are given here.

DISTRIBUTION IN THE RABBIT

4967 and 5093 were administered intravenously to individual rabbits in doses of 8 mgm./kgm. Each injection lasted approximately two minutes. Rabbits were sacrificed by injection of air at selected intervals; and blood was withdrawn by cardiac puncture, and a portion was immediately centrifuged to obtain plasma. The tissues shown in the appropriate tables were also withdrawn. Of these 'brain, spleen, pancreas, lung and heart' refer to the whole organ; 'muscle' to a sample of 2 gm. from the left hind thigh; 'fat' to perirenal fat; 'intestine' to a three-inch section of the upper duodenum; 'kidney' to the entire left kidney; and 'bile' to a sample withdrawn by syringe from the

TABLE III

Distribution of 5093 in the rat after oral doses of 80 mgm./kgm.

Time (hours)	Concentrations in mgm./l. or kgm.						
	Blood	Plasma	Liver	Lung	Spleen	Kidney	Muscle
$\frac{1}{2}$	2.79	0.79	21.4	10.8	3.97	10.7	0.938
1	2.91	0.95	26.4	12.1	8.48	11.9	2.69
$1\frac{1}{2}$	3.60	1.11	27.3	11.8	8.89	9.63	1.52
$2\frac{1}{2}$	4.36	1.25	49.6	23.4	13.4	17.6	7.19
$3\frac{1}{2}$	4.20	1.91	52.8	23.3	16.6	22.2	8.12
5	3.94	0.96	34.7	21.1	6.61	6.28	6.23
7	2.93	0.95	67.2	17.2	8.34	13.3	6.61
24	2.21	0.62	30.9	8.89	5.30	10.7	4.51

TABLE IV

Distribution of 4967 in the rabbit after intravenous doses of 8 mgm./kgm.

Tissue	Concentration in mgm./l. or kgm. after		
	20 minutes	2 hours	18 hours
Blood ...	2.74	0.525	0.191
Plasma ...	1.43	0.234	0.085
Brain ...	1.67	2.12	1.09
Muscle ...	4.75	2.67	0.90
Fat ...	0.85	2.34	0
Pancreas ...	19.1	—	2.47
Intestine ...	21.2	—	1.86
Heart ...	25.3	11.8	1.58
Liver ...	15.2	4.08	0.167
Spleen ...	20.2	10.7	0.84
Lung ...	47.0	23.5	2.35
Kidney ...	49.5	20.4	2.19
Bile ...	2.53	6.4	—

gall-bladder. The intestine was slit longitudinally, washed in three changes of distilled water, and dried on filter-paper before analysis. The analyses of fat and brain are probably subject to a somewhat greater analytical error than those of other tissues (Spinks and Tottey, 1946a; Spinks, 1947a). The results are shown in Tables IV and V.

BLOOD, PLASMA AND TISSUE CONCENTRATIONS IN THE CHICK

Each compound was administered to groups of six chicks in doses of 40 mgm./kgm. A group was sacrificed at a desired interval after dosing by withdrawing heart blood under chloroform anaesthesia. The syringe was washed with saturated potassium oxalate before each heart puncture, and the blood was delivered into a tube containing a trace of heparin. Plasma was usually lightly contaminated with haemoglobin. The results of blood and plasma analyses are shown in Table VI, those of tissue analyses in Table VII and VIII.

TABLE V

Distribution of 5093 in the rabbit after intravenous doses of 8 mgm./kgm.

Tissue	Concentration in mgm./l. or kgm. after		
	20 minutes	1 hour	18 hours
Blood ...	1.13	0.679	0.173
Plasma ...	0.525	0.292	0.082
Brain ...	2.61	0.789	0.567
Muscle ...	3.78	2.82	0.739
Fat ...	2.07	1.48	0.143
Pancreas ...	13.1	2.35	1.29
Intestine ...	24.7	1.33	1.30
Heart ...	18.8	9.99	0.543
Liver ...	11.5	5.33	0.367
Spleen ...	24.0	11.8	2.37
Lung ...	41.1	22.9	2.19
Kidney ...	48.3	8.28	0.618
Bile ...	4.79	3.08	—

TABLE VI

Blood and plasma concentrations of some homologues of paludrine in the chick after oral doses of 40 mgm./kgm.

Compound	Tissue	Concentrations in mgm./l. after								
		$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hr.	2 hr.	2 $\frac{1}{2}$ hr.	3 $\frac{1}{2}$ hr.	5 hr.	7 hr.	24 hr.
5093* ...	Blood	0.57	1.40	2.39	2.43	2.63	2.95	2.94	1.70	0.62
	Plasma	0.19	0.50	0.64	0.95	0.89	1.04	0.85	0.76	0.25
4967 ...	Blood	0.92	1.07	1.64	2.24	2.48	1.98	1.67	1.34	0.98
	Plasma	0.39	0.56	0.57	0.85	0.99	0.82	0.64	0.60	0.31
Paludrine	Blood	0.43	1.17	1.83	1.46	2.20	2.02	2.07	1.76	0.47
	Plasma	0.25	0.51	0.88	0.33	0.51	0.95	0.86	0.74	0.24
4565* ...	Blood	0.75	1.50	1.63	1.36	1.82	1.94	2.15	1.34	1.48
	Plasma	0.28	0.46	0.55	0.56	0.80	0.93	0.99	0.50	0.70
4635* ...	Blood	0.55	1.09	1.11	1.01	1.32	1.61	1.43	1.72	1.36
	Plasma	0.37	0.48	0.57	0.65	0.47	0.82	0.74	0.92	0.62

* Data are the means of two separate experiments.

TABLE VII

Distribution of paludrine in the chick after oral doses of 40 mgm./kgm.

Time (hours)	Concentrations in mgm./l. or kgm.					
	Blood	Plasma	Lung	Liver	Brain	Bile
$\frac{1}{2}$	0.43	0.25	1.45	26.2	0.995	0.98
1	1.17	0.51	11.9	56.9	1.01	8.95
1 $\frac{1}{2}$	1.83	0.88	9.55	50.2	1.86	12.6
2	2.20	0.51	23.6	57.8	2.73	5.40
2 $\frac{1}{2}$	2.02	0.95	19.7	49.8	1.74	17.8
3	2.07	0.86	10.4	52.5	5.50	10.9
5	1.76	0.74	24.4	61.4	4.92	35.7
7	0.47	0.24	18.6	20.6	4.38	15.2
24						

TABLE VIII

Distribution of 5093 in the chick after oral doses of 40 mgm./kgm.

Time (hours)	Concentrations in mgm./l. or kgm.					
	Blood	Plasma	Lung	Liver	Brain	Bile
$\frac{1}{2}$	0.57	0.19	4.90	27.6	2.12	—
1	1.40	0.50	25.5	19.8	1.12	0.61
1 $\frac{1}{2}$	2.39	0.64	28.8	19.7	—	3.85
2	2.43	0.95	20.8	44.5	1.68	5.82
2 $\frac{1}{2}$	2.63	0.89	6.12	10.4	2.20	2.75
3	2.95	1.04	—	—	—	—
3 $\frac{1}{2}$	2.94	0.85	20.2	38.2	3.94	7.75
5	1.70	0.76	16.9	33.4	4.07	5.12
7	0.62	0.25	6.81	4.82	2.37	5.05
24						

DISCUSSION

In both chick and rat oral administration of the homologues studied tends to give increasing blood concentrations with decreasing molecular weight. This trend is much more marked in the rat than in the chick, but it is clear in both and is what would have been expected from theoretical considerations and from previous work. For example, in an homologous series of 2-sulphanilamido-4,6-dialkoxypyrimidines increase in molecular weight was found to result in decreased blood concentrations when the compounds were administered orally to mice under standard conditions (Spinks, 1947*b*). Such an effect presumably depends mainly on the decrease in rate of absorption which must result from the consequences of increased molecular weight: reduced solubility (in water), and reduced rate of diffusion. However, a regular effect on blood concentration means that there must be not only a regular effect on rate of absorption, but also an equally regular effect, if any, on rate of disappearance from the blood. In the present experiments the small numbers of animals used, the dependence of an apparent rate of disappearance from the blood on the accuracy of one or two analyses (24 hours after dosing), and the influence of rate of absorption on the apparent rate of disappearance after oral administration, prevent detailed comment on this point. It is, however, an important one, since there is no doubt that the rates of metabolism of paludrine analogues are among the most important of the factors which influence their rates of disappearance from the blood (Spinks and Tottey, 1946*b*; Spinks, 1947*a*; Schmidt *et al.*, 1947; Butler *et al.*, 1947); and there may be a correlation between the extent of metabolism and therapeutic activity (see below). In so far as a conclusion can be drawn from the present data, it must be that no evidence has been obtained of any marked difference between the compounds as regards rate of disappearance from the blood.

One major difference between the rat and chick must be noted. Although the dose in the chick was half that in the rat, all compounds that were administered to both, except 5093, gave higher concentrations in the former. This amplifies the similar observation that was made when paludrine was administered over several days to rat and chick (Butler *et al.*, 1947).

There are few differences between the compounds as regards distribution in the tissues. So that the data may be viewed as a whole, the mean tissue/plasma concentration ratios of all biguanides so far examined are given in Table IX. Results from previous papers (Spinks, 1946, 1947*a*) are included. The figures were obtained by taking the arithmetic mean of all observations made on a given drug at a given dose in the same species, including those made at widely different time intervals. They are therefore only to be regarded as comparative in a very general sense, as there is no strict correlation between tissue concentration and plasma concentration; the ratio of these usually varies with the time after dosing and with other factors (cf. Marshall and Dearborn, 1946; Dearborn, 1947). Subject to this limitation, it seems that 5093 tends to be less localized in tissue than paludrine, and that paludrine tends to be more localized in rabbit than in rat tissue. The differences are not marked, and in general the biguanides are distributed similarly.

The bearing of the results on therapeutic activity may best be illustrated with respect to 5093. This inactive homologue of paludrine gives higher concentrations than the latter in chick and rat blood under standard conditions of oral administration. It is therefore unlikely that its inactivity in the chick is due to poor absorption. Although it is possibly less localized in cells than paludrine, the effect of its higher concentration in the blood

and plasma is to make the tissue concentrations of the two compounds very similar. Its inactivity is therefore probably not connected with faulty localization in the host. It has been suggested previously (Spinks and Tottey, 1946*b*; Spinks, 1946, 1947*a*) that the selective affinity of antimalarials for host tissues may be correlated with a comparable affinity for parasite cells; and this suggestion, if well founded, would mean that 5093 must reach the parasite as readily as paludrine. However, direct evidence is lacking, and this can only be a very tentative assumption. The remaining obvious reasons for inactivity are low intrinsic activity and faulty metabolism. The importance of the former is clear; the possible importance of the latter is illustrated by the suggestion of Hawking (1947) that paludrine is itself inactive but is converted by the host to an active metabolite. In the absence of any evidence from the present study that the inactivity of 5093 is due to poor absorption or faulty localization, it is reasonable to suspect low intrinsic activity or faulty metabolism, and these possibilities are being studied by the author's colleagues.

The inactivity of 4134 may be analogously ascribed to one of these two possibilities. The activities of the other compounds are somewhat similar, and the variation that does

TABLE IX

Mean tissue/plasma concentration ratios of biguanides in rat, rabbit and chick

Compound	Animal	Route	Dose (mgm./kgm.)	Mean ratio for					
				Brain	Muscle	Liver	Lung	Spleen	Kidney
Paludrine ...	Rat	Oral	80			49	14	15	20
4430 ...	"	"	80			56	34	22	33
5093 ...	"	"	80		5	38	31	8	12
Paludrine ...	Chick	"	40	6		84	29		
5093 ...	"	"	80	5		30	31		
Paludrine ...	Rabbit	Intravenous	8	5	21	57	155	97	188
4967 ...	"	"	8	8*	8*	10*	53*	23*	49*
5093 ...	"	"	8	5*	9*	15*	61*	38*	42*

* Each of these values is a mean of only three observations.

exist is not obviously associated with any of the present findings. However, one possibility may be mentioned. Therapeutic activity rises along the series 5093 to 4635 until paludrine is reached, and then falls. As blood and plasma concentrations fall continuously along the series, the results are not at variance with the hypothesis that the observed therapeutic effects are due to interaction between this continuous fall and a concomitant continuous increase in intrinsic activity.

SUMMARY

The blood, plasma and tissue concentrations attained by paludrine and a number of its homologues have been determined under standard conditions in rat, rabbit and chick. The therapeutic inactivity of the lower homologues does not appear to be due to poor absorption or low affinity for cellular structures.

In rat and chick the blood concentrations of the compounds were found to decrease with increasing molecular weight.

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THE RÔLE OF BACTERIA IN INTESTINAL AMOEBIASIS IN MAN

BY

G. T. STEWART*

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)

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Entamoeba histolytica is regularly associated with bacteria during the active stages of its life-cycle, with the notable exception of its growth in an amoebic liver abscess. In the free-living state, as in cultures, the amoeba requires bacteria or their products for its excystation, multiplication and encystation (Chinn *et al.*, 1942). As a parasite in the human or animal intestine, *E. histolytica* is intimately associated with the indigenous bacterial flora of the host, and it is probable that its pathogenicity is in some way linked to the accompanying bacteria. This idea is supported by experimental and clinical evidence. Experimental evidence suggests that, in several animal species, virulent bacteria are necessary for the development of lesions in the colon (Wenyon, 1926; Frye and Meleney, 1933; Deschiens, 1938); and clinical evidence has been derived from the fact that cases of amoebic dysentery may benefit from treatment with antibacterial agents, such as sulphonamides (Bloom, 1944) and penicillin (Hargreaves, 1945), in addition to the usual anti-amoebic drugs. A bacteriological survey of severe human infections (Stewart, 1947) failed to incriminate any common pathogen, though significant changes occurred in the proportions of the various organisms in the faeces. The absence of any single pathogen suggests, as a hypothesis, that the rôle played by bacteria may be variable in character, depending upon the virulence of bacteria already present in the bowel, rather than upon extraneous pathogens. A mechanism of this kind is demonstrable in experimental amoebic infection in the rat, in which such bacteria play a major rôle in the pathogenesis and subsequent course of the infection (Stewart and Jones, 1948a).

The purpose of this paper is to amplify some studies previously reported (Stewart, 1947) upon the element of added bacterial infection in the pathological course of amoebiasis in man.

METHOD OF INVESTIGATION

The clinical material consisted of 101 male Service cases of proven intestinal amoebiasis, comprising 35 early dysenteric cases and 66 late relapsing cases. Symptomless carriers were excluded. A group of 120 medical and surgical cases with no intestinal disorders served as controls. The following investigations were carried out.

Clinical Investigation

The clinical investigation included sigmoidoscopies, leucocyte counts, and, in some cases, blood cultures and serological tests. All the cases showed *E. histolytica* in the faeces during the period of investigation. Bacteriological cultures were made from the faeces in all the cases and in the controls. The specimens were preserved in glycerol-saline and plated on the following agar media: horse-blood, with or without 0.1 per cent. sodium azide; MacConkey; desoxycholate citrate; bismuth sulphite. The blood and MacConkey

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plates preserved the main aerobic flora; the azide plates suppressed coliform organisms but preserved gram-positive cocci (Snyder and Lichstein, 1940); the desoxycholate-citrate and bismuth-sulphite plates were used as selective media to preserve certain non-lactose-fermenters and pathogens of the *Shigella* or *Salmonella* groups. The plates were incubated aerobically at 37° C. for 24–48 hours.

In some preliminary experiments, plates were also incubated anaerobically. By this means, fusiform bacteria and gram-positive rods (*Clostridia*) were preserved. The greater part of the flora, however, was still accounted for by coliform organisms and gram-positive cocci, which are facultatively anaerobic and occurred in approximately the same proportions as in the aerobic cultures. Obligate anaerobes never attained predominance in cultures made from normal or pathological faeces in the series investigated. The present study was therefore confined to aerobic cultures.

The proportion of different organisms in the faeces was assessed by a rough numerical survey of the colonies on the non-selective media. The various organisms isolated were subcultured into peptone and carbohydrate media, for biochemical reactions, etc. Formolized broth cultures and alcoholized suspensions were prepared for serological tests.

Animal Experiments

Rats, mice and guinea-pigs were used to assess the virulence of various organisms, as described below. Rabbits were used to prepare antisera against paracolon strains.

RESULTS

General Findings

The 101 cases all showed the clinical features which are familiar in intestinal amoebiasis—acute or intermittent diarrhoea, abdominal pain and tenderness. The only common manifestations of constitutional upset were varying degrees of lassitude and loss of weight. Even in severe cases pyrexia was slight or absent, and high leucocyte counts did not occur, although dysenteric cases usually showed a mild leucocytosis, of the order of 12,000 cells per c.mm., as described by Manson-Bahr and Willoughby (1928). In some instances a leucocytosis was absent in severe relapsing cases, in spite of the presence of frank pus in the stools.

Sigmoidoscopies were performed upon most of the cases before treatment. The early dysenteric cases showed shallow ulcers with red vascular bases; occasionally yellowish necrotic foci were seen. The intervening mucosa was intact and showed little change beyond hyperaemia. In late cases, the ulcers were deep and full of muco-pus; the intervening mucosa was oedematous and intensely congested. Biopsies taken from this type of case showed that there was a wide-spread inflammatory infiltration of the mucosa. In some cases, known in the Liverpool School of Tropical Medicine as 'post-dysenteric colitis,' the entire mucosa was replaced by pyogenic granulations, which often obscured the original amoebic infection (Stewart, 1947). Post-dysenteric inflammation of this type could develop suddenly, in a few weeks, or gradually over a period of months; the amoeba could persist or disappear during its development, and anti-amoebic treatment *per se* produced little or no response. Whatever the exact mechanism, it was obvious that other factors in addition to *E. histolytica* were concerned in the aetiology of this type of colitis.

The exudates from the diseased colon in early dysenteric cases showed vegetative forms of *E. histolytica*, with some loose mucus and blood derived from the ulcers. Micro-

scopically, a few leucocytes and macrophages were present, but there was no 'pavement' of cells and faecal matter was freely admixed. Exudates of this type gave an acid reaction (pH 5), or, if faecal material was present, a weakly acid or neutral reaction. A strongly acid reaction was usually associated with an overgrowth of *Bacterium aerogenes*. In late cases, frank pus appeared in the faeces and innumerable leucocytes could be seen microscopically; with the appearance of pus, the reaction of the exudate became neutral or alkaline, and, as will be seen later, this was associated with an increase in non-lactose-fermenting bacteria. By analogy with bacillary dysentery (where the fresh exudate is invariably alkaline) it seemed likely that the alkaline reaction was derived from the non-lactose-fermenters (or the associated tissue reaction), and not vice versa.

TABLE I

Relative distribution of organisms in the faeces of controls, cases of early amoebic dysentery and late relapsing amoebiasis

Group	No. in group	Proportions	<i>Bact. coli</i>	<i>Bact. aerogenes</i>	Non-lactose-fermenters	Enterococci
Controls	120	Present Predominant	120 (100%) 114 (95%)	10 (8.3%) Nil	23 (19%) 3	74 (61.6%) 2
Early amoebic dysentery	35	Present Predominant	See text 6 (17%)	29 29 (83%)	See text	
Relapsing amoebiasis	66	Present Predominant	63 (95.4%) 47	8 (12.1%) 2	21 (31.8%) 3	59 (89.4%) 7

TABLE II

Differences in the distribution of certain organisms in the faeces of controls, cases of early amoebic dysentery and late relapsing amoebiasis

Group	No. in group	<i>Bact. aerogenes</i>	Enterococci	Paracolon	<i>Bact. morgani</i>	<i>Proteus</i>	<i>B. alcaligenes</i>
Controls	120	18	74	10	7	3	2
Relapsing amoebiasis	66		59	14	7	2	1
Early amoebic dysentery	35	29	See text				
P		0.01	0.01	0.02	0.2 > P > 0.1		

Blood cultures were taken from 10 severe cases. The results were negative. Manipulations, such as sigmoidoscopy, did not cause a bacteraemia.

Bacteriological Findings

When wet and stained smears of faeces were examined, the bacterial flora, in normal and in dysenteric specimens alike, was found to be abundant and varied. Gram-negative rods were usually predominant, varying greatly in size and in staining properties. Fusiform bacilli, yeast-like cells, spirilla, gram-positive rods and cocci were all present.

In contrast to this, cultures made from faeces were comparatively simple to interpret. The relative distributions of organisms in the cases and controls are shown in Table I and the differences are analysed in Table II.

All the controls showed numerous colonies of *Bact. coli*, and in a majority (95 per cent.) this organism was predominant. Enterococci were present in 61 per cent., but were rarely predominant. In 27 per cent. of the normal specimens, a few colonies of non-lactose-fermenters or *Bact. aerogenes* were present, and in isolated instances (2.5 per cent.) the non-lactose-fermenters outnumbered *Bact. coli*.

The 101 cases of intestinal amoebiasis showed the following changes:

1. Early dysenteric cases (usually in their first known attack of dysentery) showed a significant difference from the controls in the increased incidence of *Bact. aerogenes*. In many cases this increase amounted to a complete overgrowth by *Bact. aerogenes* of all the other bacteria present, and where this occurred no final opinion could be reached regarding the relative distribution of the other organisms. Stained smears taken from the plates and direct smears made from the faeces showed that enterococci were usually abundant.

2. Late relapsing cases, with chronic lesions in various stages of activity, showed an increased number of non-lactose-fermenters. In broad terms, this indicated that the bacterial flora was more mixed in these cases than in the controls, but statistical analysis showed that the only organism of which the prevalence was significantly increased was paracolon (Table II).

3. Both groups showed a significant increase in the presence of enterococci; quantitatively, the proportion of enterococci was greater in liquid than in solid stools.

4. Coincident infections with *Shigella* or *Salmonella* organisms were absent in this series. In this connection it is relevant to mention that, in a corresponding series studied in the tropics (Ceylon), four out of 100 cases showed simultaneously the presence of *Shigella* (three *flexneri*, one *sonnei*) along with *E. histolytica* (Stewart, O'Meara and Kershaw, 1948). In acute cases seen in the tropics the overgrowth of *Bact. aerogenes* referred to above was usually very marked.

Serological Reactions

From 30 cases of amoebiasis, the patient's serum was mixed with formalized and alcoholized suspensions of *Bact. coli* isolated from his own faeces. Five cases showed 'O' type agglutination to a titre of 1:50. One of the five gave 'H' agglutination to a titre of 1:125. The remainder gave negative results.

Control experiments were performed by testing pooled or individual sera from 10 groups of 10 healthy individuals against strains of *Bact. coli* isolated from normal stools and from stools containing *E. histolytica*. This investigation showed that positive reactions could occur at titres of 1:25 and occasionally of 1:50 ('O' type agglutination), though not higher. Furthermore, some strains of *Bact. coli* isolated from dysenteric stools were agglutinated by normal sera, though not by the patient's own serum. This contradiction showed that the possibility of accidental agglutinations and cross-reactions excluded any pathological significance in the serological reactions of the patients with amoebiasis.

Paracolon bacteria and the other gram-negative bacteria isolated were also tested against sera from patients and controls. One case showed titres of 1:50 ('O') and 1:200 ('H') against his own paracolon organism. *Proteus*, *Bact. morgani*, *Bact. aerogenes* and *B. alcaligenes* were not agglutinated.

Of the series investigated, therefore, only two showed agglutination titres of importance. It can be concluded that cases of amoebiasis do not commonly develop agglutinins against the predominant members of the intestinal flora, although a reservation must be

made concerning severe cases of post-dysenteric colitis (see above), where positive reactions are relatively more common.

Notes on Individual Bacteria

Paracolon. The 24 organisms thus designated were late (48 hours) or non-lactose-fermenters. All the strains fermented glucose and mannitol with acid and gas production. The majority belonged to groups A or D (Dudgeon and Pulvertaft, 1927), corresponding to groups I and II of Seviitt (1945). Seviitt has shown that 75 per cent. of his group I strains were serologically identical, and that they all contained minor antigens common to the *Shigella* group. The occurrence and probable importance of strains with *Shigella* or *Salmonella* antigens has also been discussed by Felsenfeld and Young (1945). Two of the strains isolated from the present series of cases possessed *Salmonella* 'O' antigens I and II, and most were motile. Such strains were differentiated from *Salmonella* by the production of indole. The usual IMViC pattern among group I and II bacteria was (+ + — —). Where the methyl-ered reaction was positive the Vosges-Proskauer reaction was always negative, and vice versa. Group I and II strains were virulent to rats, mice and guinea-pigs, and were shown to aggravate an experimental amoebic infection in rats (Stewart and Jones, 1948a).

Enterococci. In the present studies, heat-resistant gram-positive cocci isolated selectively on the azide media were classified as enterococci. The majority were lanceolate diplococci which fermented glucose, mannitol and saccharose and grew at pH 6.0 or in 6.5 per cent. NaCl. No β -haemolysis occurred in horse-blood agar. It is probable that most of these organisms were *Streptococcus faecalis*. Dible (1921) has shown that, normally, enterococci are of low virulence. Strains isolated from dysenteric cases and controls were equally non-virulent to rats, mice and guinea-pigs by the oral or intraperitoneal routes. Cultures made from different levels in the rat's intestine showed that the organism was more prevalent in the small than in the large intestine. When diarrhoea was provoked by the use of saline purgatives, enterococci became relatively more numerous in the stools. It is probable therefore that prevalence of *S. faecalis* in dysenteric stools was a physiological consequence of intestinal hurry.

In a few instances, staphylococci were observed in cultures, but there was no significant difference in their occurrence in the control and in the dysenteric groups.

DISCUSSION

The results show that the bacterial flora of the colon may exhibit three abnormal deviations in intestinal amoebiasis: the predominance of *Bact. aerogenes* in the early dysenteric stage; the increased frequency with which paracolon organisms may be isolated in the later stages of the relapsing disease; and the relative increase in the proportion of enterococci in diarrhoeic stools at any stage. Severe relapsing infections are associated with a pyogenic inflammatory response in the colon, and pus appears in the stools. In spite of these local changes, which are often extensive, there are comparatively few signs of systemic reaction, as evidenced by the comparative fitness of the patient and by the absence of fever or high leucocytosis, bacteraemia or agglutinins in the serum.

The appearance of numerous enterococci in the faeces appears to be a physiological phenomenon, due to the rapid passage of the contents of the small intestine in states of diarrhoea. Investigations described elsewhere (Stewart, Jones and Rogers, 1948) show that certain strains of enterococci of the *S. faecalis* group inhibit *E. histolytica* *in vitro* and *in vivo*; *in vitro* this effect depends upon the production of one or more toxic metabolites from media containing glucose or related carbohydrates. Human strains of *S. faecalis* isolated from dysenteric cases in the present series show similar activity. It is therefore possible that the prevalence of such strains in the active stages of amoebiasis may be associated with a process of natural remission.

The increase in *Bact. aerogenes* in early acute cases is not easy to explain. This organism may be present in normal human faeces, the frequency of its occurrence varying in different communities from 13 per cent. (Kempny, 1946) to 40 per cent. (Mollari *et al.*,

1939). In the controls of the present series its incidence was 8·3 per cent., but, as Bardsley (1934) has shown, it can be isolated with greater frequency by the use of enrichment techniques. It is clear, however, that, in spite of the considerable normal variation in its incidence, *Bact. aerogenes* does not normally attain predominance in the faeces. Some increase may occur after saline purgation, but the present studies suggest that predominance is associated with the acid reaction of the exudate in amoebic dysentery. The organism is not observed in the acute stage of bacillary dysentery, where the exudate is invariably alkaline, and it is less regularly isolated in late cases of amoebiasis or in post-dysenteric colitis where pus appears. When the cultures are fed to stock rats not carrying the organism, *Bact. aerogenes* fails to gain prevalence in the intestine—whether by excretion, destruction or modification of its characters is not clear. Cultures fed to young rats experimentally infected with *E. histolytica* fail to aggravate the infection (Stewart and Jones, 1948b). The balance of evidence, therefore, is that the increase in *Bact. aerogenes* in acute amoebic dysentery is an associated phenomenon, and that it does not necessarily represent an invasion by this particular organism of the amoebic lesions.

Paracolon bacteria have been investigated for pathogenicity by several workers. Glynn *et al.* (1917) isolated organisms described as 'indole-positive paratyphoid bacilli' from enteritis convalescents during the 1914–18 war, but found no evidence of pathogenicity. Since then, however, evidence has been found that organisms corresponding to those described by Glynn *et al.* may assume a pathogenic rôle in certain bowel disorders. Dudgeon and Pulvertaft (1927) showed that slow-lactose-fermenting coliforms could often be isolated in pure culture from cases of acute diarrhoea; they classified such organisms, and showed that the majority of the presumptively pathogenic strains were biochemically and serologically uniform. Stuart *et al.* (1943) considered that paracolon was intermediate in biochemical and antigenic structure between normal *Bact. coli* and the *Salmonella* group, and that it might act as a pathogen in conditions of mild enteritis. This finding may be correlated with the fact, familiar to most bacteriologists, that paracolon bacteria appear in the faeces in large numbers during the convalescent stages of acute *Salmonella* or *Shigella* infections (Weil, 1947). Sevitt (1945) reclassified the paracolon group and showed that certain members had an increased incidence among infants with infectious enteritis; many of his strains showed similarities in antigenic structure to the dysentery bacilli. Such strains produced experimentally an enterocolitis in kittens, in which the specific organism could be recovered from the faeces. Ferguson and Wheeler (1946) isolated two paracolon strains antigenically related to *Shigella paradysenteriae*, and Barnes and Cherry (1946) found paracolon in the stools of 12 out of 17 cases in an outbreak of gastro-enteritis in a United States naval hospital. Thus there is evidence in the literature that paracolon is associated with certain conditions causing diarrhoea. An attempt has been made to investigate further its rôle in amoebiasis by feeding or injecting specific paracolon bacteria, isolated from human cases, to young rats experimentally infected with *E. histolytica* (Stewart and Jones, 1948b). This resulted in an increase in the severity of the lesions, and identical strains could be recovered from the infected bowel and, in some instances, from the blood or peritoneum. When fed to control rats, paracolon provoked hyperaemia in the ileum and caecum and could in some instances be recovered from those sites. Injected intra-peritoneally, these strains, in smaller doses, induced a fatal bacteraemia but showed no specific tissue-fixation. Thus, under experimental conditions paracolon behaves as a facultative pathogen which profoundly influences the course and severity of lesions initiated

by *E. histolytica*. In this sense Koch's postulates are fulfilled by recovery of the identical strains.

It may be concluded, therefore, that paracolon bacteria are potentially pathogenic when the intestine is diseased by amoebic infection, and that their presence, natural or otherwise, in a proportion of individuals introduces an added element of bacterial infection in amoebic lesions. Since the strains isolated from such cases are not always biochemically or serologically identical, the precise limitations of the pathogenicity of the organism cannot as yet be defined.

Other non-lactose-fermenters isolated from cases of amoebiasis include *Bact. morgani*, *B. faecalis alcaligenes* and *Proteus*. The pathogenicity of these organisms has been repeatedly investigated (Morgan and Ledingham, 1909; Trawinski and György, 1918; Bengtson, 1919), and Wilson (1929), reviewing their position, found no convincing evidence of their pathogenicity in conditions of enteritis. In the present series there was no significant difference in the incidence of these organisms between cases of amoebiasis and controls. This finding in itself does not mean that the organisms could not assume a pathogenic rôle, but it does suggest that their rôle was inconspicuous.

In many of the severe relapsing cases, the aerobic bacterial flora contained only *Bact. coli* and enterococci. Strains of *Bact. coli* isolated from such cases proved highly virulent when fed to rats experimentally infected with *E. histolytica*; evidence was also obtained that strains of *Bact. coli* native to the rat's intestine played a rôle in every experimental infection (Stewart and Jones, 1948a).

In the interpretation of these findings, the absence of recognized pathogens of the *Shigella* group is of importance. Such organisms may be found among cases diagnosed in India (Acton, 1933; Marriott, 1945), though their presence is not necessary for the initiation of amoebic dysentery (Stewart, O'Meara and Kershaw, 1948). If unidentified anaerobes be excluded, the element of bacterial infection in the present series of cases must therefore reside in the various organisms described above. This means that added or 'secondary' bacterial infection in amoebiasis depends upon the capacity of organisms already present in the bowel to invade lesions established in the first instance by *E. histolytica*. The severity and course of the resulting disease depends upon the potential pathogenicity of indigenous strains of *Bact. coli* and upon the presence, natural or otherwise, of facultative pathogens such as paracolon.

SUMMARY

Cases of intestinal amoebiasis in the chronic relapsing stage show pyogenic inflammatory changes in the mucosa of the colon. This pathological process is essentially a local one; a systemic reaction is uncommon.

In the course of the disease alterations occur in the relative distribution of organisms in the faeces.

The early dysenteric stages are characterized by a tendency towards overgrowth of the other organisms by *Bacterium aerogenes*, a process to which no pathological importance is ascribed.

Enterococci are prevalent in diarrhoeic specimens at any stage. This probably results from the rapid passage of the contents of the small intestine. The majority of enterococci are non-virulent, and the metabolites produced by certain strains are toxic to *Entamoeba histolytica*.

In chronic relapsing cases the incidence of paracolon bacteria is increased ; evidence is adduced to show that these organisms assume a pathogenic rôle in a proportion of such cases.

It is suggested that the inflammatory reaction in the colon in severe relapsing cases is largely attributable to added bacterial infection, dependent upon the virulence of the indigenous bacteria and upon the occasional presence of potential pathogens, such as paracolon.

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SYLVATIC PLAGUE IN SOUTH AFRICA : HISTORY OF PLAGUE IN MAN, 1919-43*

BY

D. H. S. DAVIS

(From the Plague Research Laboratory, Department of Health, Union of South Africa)

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INTRODUCTION

The history of the introduction of plague into South Africa, and of its subsequent spread into the interior to become enzootic in wild rodents, is given by Mitchell (1927). The expansion of the original sylvatic foci in the north-western Orange Free State and midland districts of the Cape Province has been responsible for the creation of the wide enzootic area which now covers more than half of southern Africa from the Cape to Barotseland in Northern Rhodesia. Sylvatic plague flourishes among burrowing rodents inhabiting rolling semi-desert country, and it is in keeping with the other large sylvatic plague regions of the world, such as the pampas of South America and the prairies and steppes of western America and Russia, that the enzootic plague region in southern Africa should comprise the karroo, the high veld and the Kalahari Desert—that is, the drier part of the subcontinent.

Within the Union plague has failed to establish itself in the northern or eastern Transvaal, Natal, the coastal region of the Cape Province between Port Elizabeth and Cape Town, or to the north of Cape Town in the low-lying western Cape Province. These areas have remained free, in spite of being exposed to the risk of infection from time to time.

Plague in man derived from a sylvatic source takes the form of sporadic outbreaks in rural areas. Farm-workers are open to the greatest risk of infection. It is rare for infection to be acquired directly from the small wild rodents which act as the primary reservoir of plague, but it has sometimes been acquired as a result of handling the larger rodents (hares, etc.) which have become secondarily infected. Most infections are contracted in farm huts and out-buildings infested with domestic rodents which have become secondarily infected from the primary reservoir in gerbils (Fourie, 1938). The common gerbils of the genus *Tatera* (*brantsii* and *schinzi*)† form the primary reservoir in the high veld and Kalahari and namaqua gerbils (*Desmodillus auricularis*) in the karroo. The semi-domestic multimammate mouse (*Mastomys coucha*) is the intermediary between the primary gerbil reservoir and man. The house-rat (*Rattus rattus*) brings infection into even closer contact with man than the multimammate mouse. The risk of plague to man is greatest in areas where one or both of these species commonly frequent farm buildings, especially when they are abundant and in close contact with wild-rodent colonies during an epizootic. It is typical at the time of a human outbreak to find that the wild-rodent colonies have died out six months or a year previously and that the domestic rodents have almost completely disappeared. In interpreting the course of events in the wild-rodent reservoir in the

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† The affinities of the South African *Tatera* are outlined by Davis (1948).

light of the incidence of plague in man one must guard against assuming too close a time-correlation between the primary epizootic and the outbreak of plague in man. Man is at risk of infection during the summer 'plague season,' whereas rodent epizootics, whether in wild or domestic species, do not show any marked seasonal incidence; furthermore, major epizootics can sweep through the wild-rodent populations without giving rise to a single case in man, if the 'transmissive' factors, such as abundance of domestic rodents and fleas, are weak or lacking.

At first sight there is nothing to prevent the gerbil-man chain of infection from bringing about urban outbreaks. The disastrous consequences of such an event are ever in the minds of public health authorities throughout the sylvatic plague region. It is, however, a matter of historical fact that there has been only one urban outbreak of any importance since 1912—that at Port Elizabeth in 1938 (Union of South Africa, 1938, 1939).

Plague in man derived from a sylvatic source has thus a rural setting in South Africa, and its history can be followed almost from farm to farm. The object of this paper is to record the history of outbreaks in man, in order to show how far the geographical distribution of human plague can be used to interpret the establishment and expansion of the sylvatic plague area, and as a pointer to the factors determining its limits.

SOURCE OF RECORDS AND MAPPING METHOD

The account which follows is drawn from one source, namely, the notifications of plague outbreaks appearing in the weekly health bulletin of the South African Department of Public Health since its establishment as a separate department in 1919. The bulletins are published as a general government notice in the *Government Gazette* (Pretoria). The notice gives the district and locality (and sometimes its location) from which outbreaks have been reported during the preceding week. The information given in the bulletin has been checked and supplemented by reference to old departmental files and case-sheets (when available). More than 900 outbreaks were notified during the period between July, 1919, and June, 1943 (the period under review), of which 95 per cent. have been located. Owing to defects in the records some of these may have been inaccurately determined, but it is considered that the 5 per cent. unlocated outbreaks or those which may have been wrongly located do not materially affect the results.

Hereafter a 'plague'-year or period will be printed in italics, to avoid confusion with the calendar year. The plague-year is taken from one mid-winter (July 1st) to the next (June 30th), as the incidence of plague is highest in man in the summer. Thus *1919-25* refers to the period from July 1st, 1919, to June 30th, 1925, and *1923-24* refers to the twelve months beginning July, 1923. An outline map on the scale 1 : 4,000,000 was drawn in the office of the Director of the Trigonometrical Survey and was printed in black with a superimposed latitude-longitude grid (unit squares of 15' \times 15' of latitude and longitude) in blue. The square formed by one degree of latitude and longitude was thus divided into 16 small 'quarter-degree' squares. The position of a locality was pin-pointed on a large-scale map (Topo-Cadastral 1 : 250,000) and given a 'locus' defined by a code indicating the quarter-degree square in which it fell. The code-map reference 2829 Ac, for example, signifies that a locality is within south latitude 28° 15'-29' and east longitude 29° 0'-14' (see diagram, page 209).

With this system of plotting, the localities are automatically centred and evenly distributed on the map, permitting clearer exposition. Each circle drawn within the grid 'unit

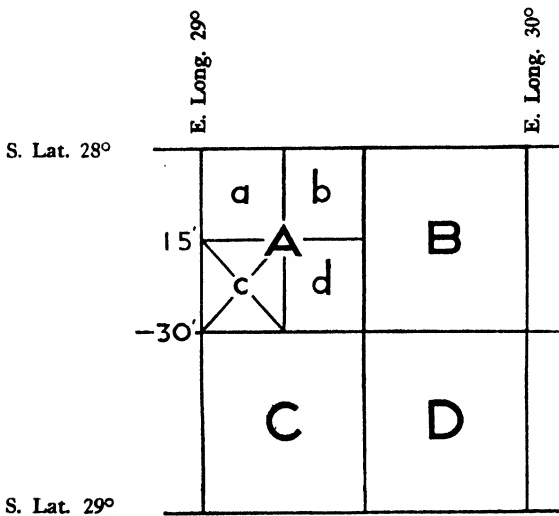
covers an area approximately 15 miles square and represents the occurrence of one or more outbreaks at one or more points within the unit area.

HISTORY OF PLAGUE

INTRODUCTORY, 1899–1919

Before turning to the examination of the bulletin records over the period 1919–43, a brief description of the events leading up to the situation in 1919, taken from Mitchell (1927), will be given and illustrated by a map (fig. 1).

The murine phase (i.e., domestic-rodent plague in urban areas) came to an end in 1912. The first sign that the disease had entered on a new phase—the sylvatic phase (i.e., wild-rodent plague in rural areas)—was the series of outbreaks on remote farms in the Cape midlands in 1914–15. A wild-rodent origin was suspected but was not proved until 1921 (Mitchell, 1921).



The course of events from the time of the first recorded case in South Africa at Middelburg, Transvaal, (a contact from Lourenço Marques) until June, 1919, is illustrated in fig. 1. The main centres of murine and human plague outbreaks are shown as squares: the large black squares mark the localities which experienced major prevalences of murine and human plague; the small black squares denote minor prevalence in rats, sometimes associated with human cases; and the open squares mark cases contracted from a murine focus elsewhere. The open circles mark the approximate localities where the first sylvatic foci were established.

The primary sylvatic foci were established at a number of points in three rather widely separated areas—the south-western Transvaal and north-western Orange Free State, the Cape midlands, and the Uitenhage district near Port Elizabeth. Each owed its infection in the first place to the spread of plague by rats and fleas in rail and other forms of traffic from the infected ports. It will be shown below that the subsequent development of the

primary foci is consistent with the view expressed by Fourie (1938) that these were independently established and were not formed by spread of plague in wild rodents from the coast.

INCIDENCE OF PLAGUE DURING THE 24 YEARS 1919-43

The number of outbreaks and cases of plague are listed in the accompanying table. There have been two major epidemic periods: in the years 1923-24-25 (217 outbreaks)

TABLE
Outbreaks of plague in man in South Africa, 1919-43

Plague-year	No. of outbreaks	No. of cases*
1919-20	1	70
1920-21	11	
1921-22	5	
1922-23	1	2
1923-24	165	372
1924-25	52	112
Totals for the years 1919-25	235	556
1925-26	29	71
1926-27	38	75
1927-28	15	39
1928-29	20	65
1929-30	53	145
1930-31	33	71
Totals for the years 1925-31	188	466
1931-32	8	22
1932-33	18	31
1933-34	12	39
1934-35	138	290
1935-36	123	253
1936-37	31	52
Totals for the years 1931-37	330	687
1937-38	15	70
1938-39	27	77
1939-40	29	47
1940-41	36	90
1941-42	31	79
1942-43	27	77
Totals for the years 1937-43	165	440
Totals for the years 1919-43	918	2,149

* 1919-23 from Mitchell (1927); 1923-43 from the Union of South Africa (1924-43).

and in 1934-35-36 (261 outbreaks). The first epidemic was associated with a wide-spread epizootic in the northern Orange Free State and Cape midlands (Union of South Africa, 1924, 1925), and the second with a major epizootic throughout the Orange Free State (Union of South Africa, 1935, 1936). In 1929-30-31 (86 outbreaks) epizootic conditions prevailed particularly in the northern Free State and in the karroo areas of the Cape Province

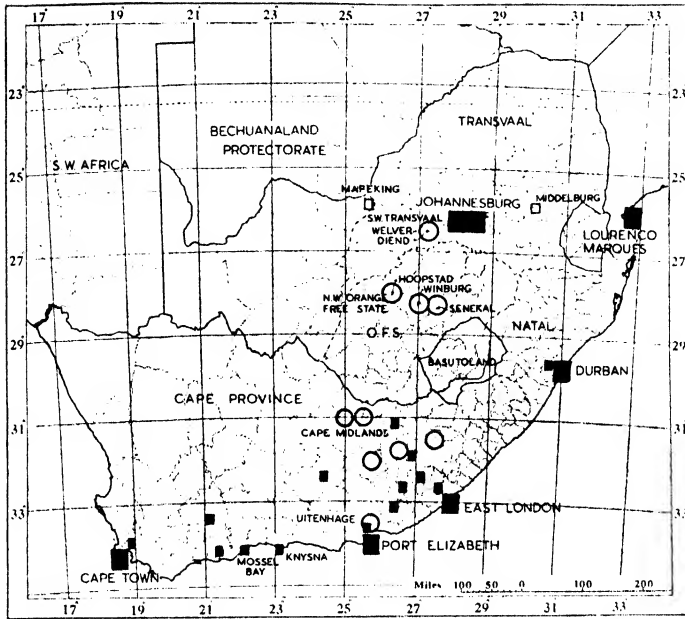


FIG. 1. 1899-1919: The introduction and spread of plague (data from Mitchell, 1927). Large black squares indicate major prevalences, and small black squares minor prevalences, of murine and human plague. Open squares indicate cases in man contracted from a murine source elsewhere. Open circles show the approximate localities where the primary sylvatic foci were established.

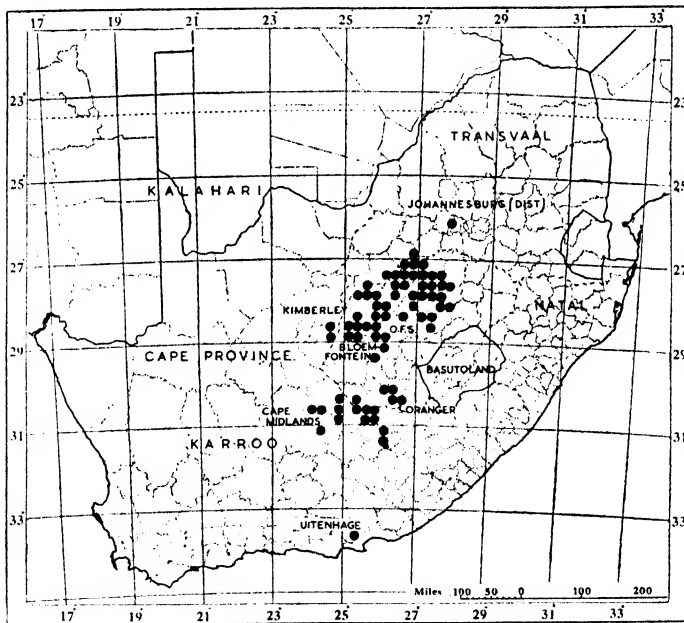


FIG. 2. 1919-25: Expansion of the primary sylvatic foci. Distribution of human outbreaks (see text for plotting method).

(Union of South Africa, 1930, 1931). The peak or epidemic years, at any rate between 1919 and 1937, have been at 5-6-year intervals.

This provided a natural division of the 24-year period into four, each of six years' duration, which, besides having epidemiological significance, is convenient for mapping the geographical distribution of the outbreaks.

1919-25 : Expansion of the Primary Sylvatic Foci (Fig. 2)

North-Western Orange Free State. The known foci in the districts of Hoopstad, Winburg and Senekal gave rise to a further series of outbreaks. The enzootic area evidently expanded to Kimberley in the west, to Bloemfontein in the south, and eastwards across the northern districts of Bothaville, Kroonstad and Vredefort, bringing a series of outbreaks in its wake. Outbreaks occurred on both sides of the Vaal River near Bothaville.

Cape Midlands. The districts in which the first outbreaks took place in 1914-15 were not all involved (cf. figs. 1 and 2). It appears that the rodent populations of the southerly districts did not experience as severe an epizootic as those further north. The impetus of the epizootic preceding the outbreaks in the north carried the infection across the Orange River into the southern Orange Free State and westwards towards the western karroo (see 1925-31 below).

Southern Transvaal. There are two possible explanations of the isolated outbreak on the outskirts of Johannesburg. In 1918 an outbreak occurred at Welverdiend in the adjoining district of Potchefstroom, and plague may have spread thence to the Reef. On the other hand, it is remotely possible that plague had been enzootic since the 1904-5 epidemic in Johannesburg and other Reef towns.

Uitenhage District. Eight farms had been involved in outbreaks in 1916. The series during the 1919-25 period was due to recrudescence of infection.

Summary. The centres of outbreaks during 1919-25 tally closely with the original sylvatic foci and support the view that these initial foci were independently established and expanded as a result of continued spread of plague among wild rodents.

1925-31 : Westerly Expansion Throughout the Karroo and Kalahari (Fig. 3)

Northern Orange Free State. The eastward extension across the northern districts of the Orange Free State continued into the districts of Lindley and Heilbron, but was there brought to a stop (cf. figs. 3, 4 and 5). Infection was carried across the Vaal River and became established in the Vereeniging district of the southern Transvaal. In addition, there were a number of sporadic outbreaks in enzootic areas. The invasion of the Heilbron district gave rise to a serious epidemic. Plague reached the gerbil-infested parts of the district and then spread from one farmstead to another, in more or less gerbil-free country, by the agency of domestic rats (*R. rattus*) (Union of South Africa, 1930).

Southern Orange Free State and North-Western Cape. Further extension south of Bloemfontein from the point reached after the 1919-25 expansion of the original focus in the north-west nearly brought about a coalescence with the Cape midlands focus. Further extension to the west of Kimberley from the same source (north-western Orange Free State) into the Kuruman district and the southern Kalahari carried the infection across the borders of the Union into South-West Africa and the Bechuanaland Protectorate to Ovamboland and Ngamiland (Fourie, 1932).

Cape Midlands. Sporadic recrudescence outbreaks in the original 1914-15 focus were associated with sporadic outbreaks at isolated points throughout the greater part of the

karroo as far west as Calvinia. The factors which are responsible for bringing about human infections are not so strong in the karroo as in the Orange Free State. For example, *R. rattus* and *Mastomys coucha* are unknown in the central and western karroo, the house-mouse (*Mus musculus*) being the only domestic rodent; in consequence, contact with infected wild rodents or their fleas (e.g., in the nest shelters of karroo rats (*Myotomys*), which are gathered for firewood) or hares (*Lepus capensis*) is the chief means of infection to man.

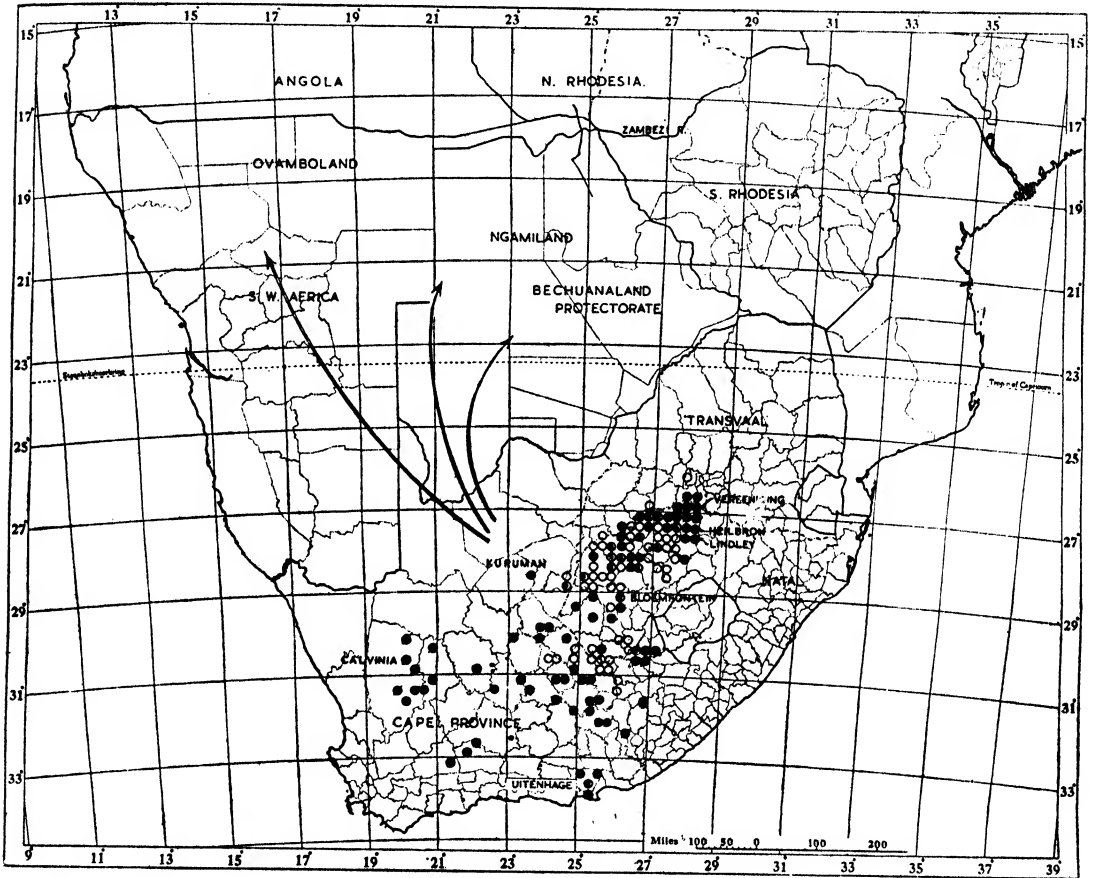


FIG. 3. 1925-31: Westerly expansion through the karroo and Kalahari. Open circles indicate outbreaks during 1919-25. Black circles indicate outbreaks during the current period, some in new localities (full circle), some in old localities (small black dot inside open circle). The arrows indicate the probable extent of spread outside the borders of the Union of South Africa.

Uitenhage District. There was recrudescence in and further expansion of the original focus.

Summary. Outbreaks were mainly in new areas, as a result of major extensions of the enzootic area in the karroo and northern Free State. They spread across the Union border into South-West Africa and Bechuanaland. Recrudescence outbreaks occurred on a small scale.

1931-37 : Epidemic in the Orange Free State (Fig. 4)

Orange Free State. General recrudescence occurred in the northern districts. No further spread took place to the east from Heilbron, in spite of wide-spread recrudescence infection. There was recrudescence and further extension of the enzootic area in the southern Free State. Epidemic conditions prevailed throughout the province as a result of a major epizootic following seasons of abnormal rainfall, during which the wild-rodent

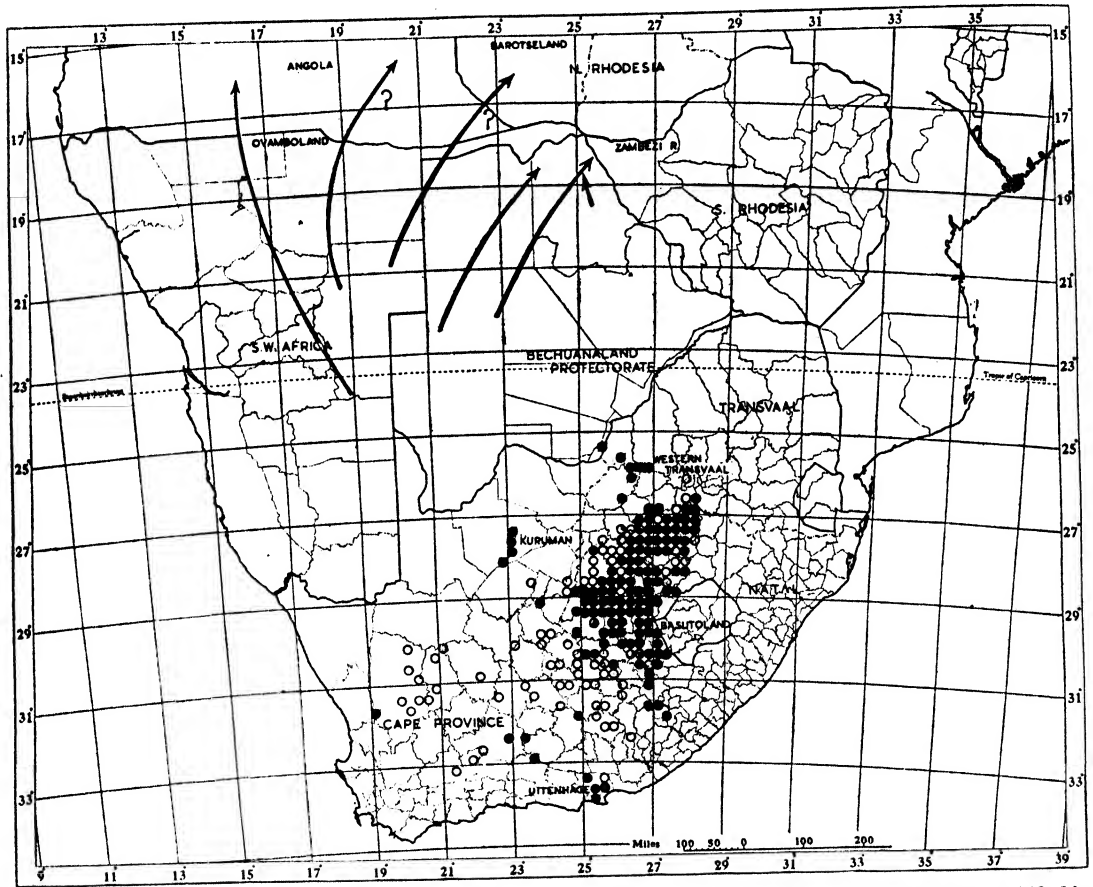


FIG. 4. 1931-37 : Epidemic in the Orange Free State. Open circles indicate outbreaks during 1919-31. Black circles indicate outbreaks during the current period, some in new localities (full circle), some in old localities (small black dot inside open circle). The arrows indicate the probable extent of spread outside the borders of the Union of South Africa.

population reached a very high population density (Union of South Africa, 1935, 1936). Plague spread across the Basutoland border from the south-eastern districts into the foothills of the Drakensberg Mountains.

Western Transvaal and Bechuanaland. The series of outbreaks in the high veld of the western Transvaal appear to have originated by extension of the enzootic area in the southern and south-western Transvaal. Plague is believed to have spread from the western Transvaal

into the south-eastern districts of southern Bechuanaland, and to have coalesced with the enzootic area established earlier during the first Kalahari invasion (Fourie ; see Davis, 1946) in Ngamiland.

Cape Midlands. The south-easterly spread towards the Transkeian territories continued, but was limited to the Glen Grey district.

Uitenhage District. There were recrudescence outbreaks and further extension of the infected area.

Summary. The epidemic of plague throughout the Orange Free State was preceded by coalescence of the karroo (Cape midlands) and high veld (northern Orange Free State) foci of infection in the southern districts of the Orange Free State ; it spread across the border of Basutoland. An expansion of the enzootic focus in the south-western Transvaal gave rise to a series of outbreaks in the western Transvaal high veld and on the borders of southern Bechuanaland, whence infection is believed to have spread northwards, to coalesce with the Ngamiland enzootic area, and to have carried the enzootic area up to the Zambezi River.

1937-43 : Recrudescence in Hyperenzootic Areas (Fig. 5)

The general impression made by the history of plague outbreaks during the period 1937-43 is that by 1937 enzootic plague had reached its limits within the borders of the Union, and that the danger of human infections was much reduced, except in three limited areas—the northern Orange Free State, the Glen Grey district and the Uitenhage district—where recurrent epizootics gave rise to the majority of outbreaks in the Union. This may indicate that the rodent populations of the karroo areas in the Cape Province and southern Orange Free State had been so decimated that recovery in population density to the levels reached before the enzootic plague era was prevented by sporadic outbreaks of epizootic plague on a small scale. A repetition of the events of 1935-36 may be expected in the future, after a period of heavy rains has provided abundant food to induce rapid multiplication of rodents, both wild and domestic. Events in the three hyperenzootic areas mentioned above were as follows.

Northern Orange Free State. Outbreaks were wide-spread throughout the district of Bothaville, Kroonstad, Vredefort and Heilbron as a result of recurrent epizootics.

Glen Grey District. This focus—an offshoot of the original Cape midlands focus—was very active. There were a number of outbreaks due both to recurrent epizootics in the Glen Grey district and to an extension of the infected area to the adjoining St. Marks district in the Transkeian territories. The enzootic area did not extend across the range of hills separating this part of the St. Marks district (Qamata Basin) from the other districts to the east.

Uitenhage and Port Elizabeth Districts. The prevalence of plague in the Uitenhage enzootic area appears to have been the source of infection to the domestic-rat populations in the suburbs of Port Elizabeth (Union of South Africa, 1938, 1939) which gave rise to a human epidemic.

Summary. By 1943 there was evidence of possible further expansion of the enzootic area at one point only—the Glen Grey-St. Marks focus, which threatened to extend into the heavily populated Transkeian territories. On the evidence of the history of human plague, the focus in the Uitenhage district appears to be isolated from the main enzootic area.

CONCLUSIONS

The main conclusion to be drawn from this study is that the history of plague in man reflects fairly accurately the history of the spread of plague in wild rodents. This is most evident in the hyperenzootic areas, such as the northern Orange Free State, where outbreaks in man follow rodent epizootics with greater regularity on account of the relatively closer contact of man with secondary infections in domestic rodents. Elsewhere, apart from the two other hyperenzootic areas in the Glen Grey-St. Marks districts and the Uitenhage-

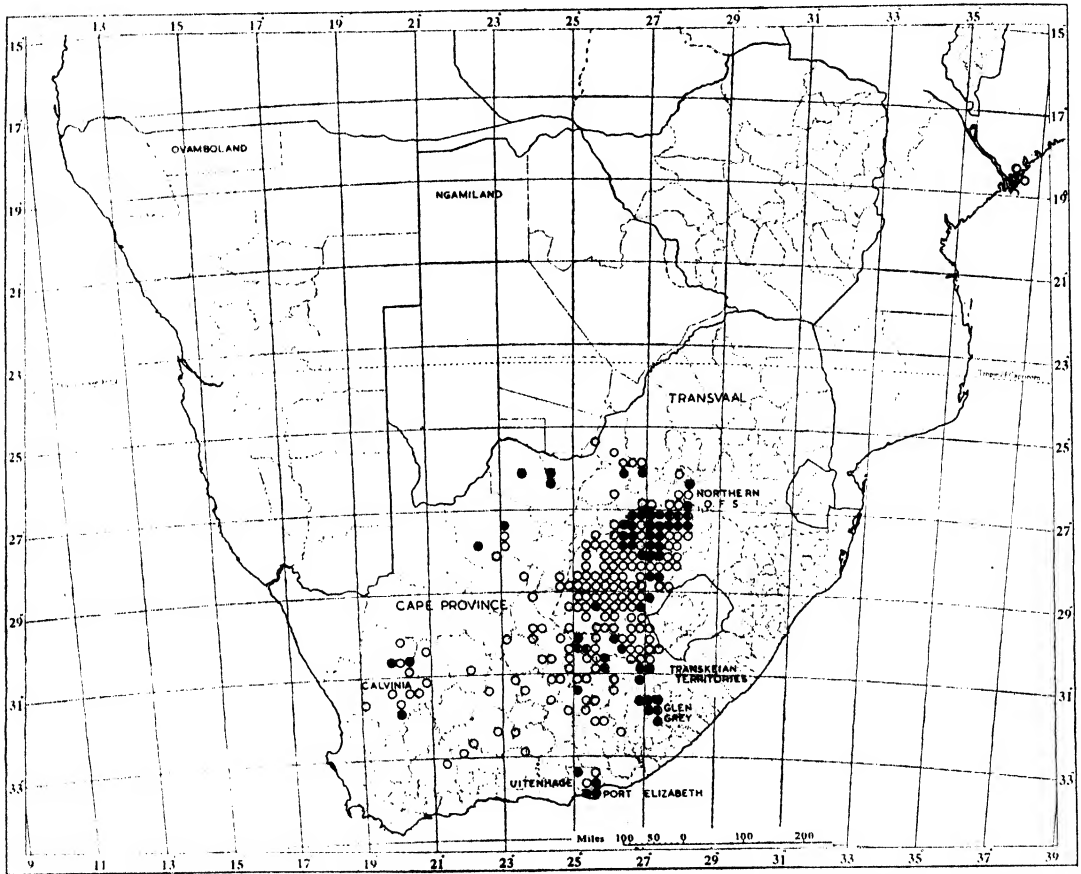


FIG. 5. 1937-43: Recrudescence in hyperenzootic areas. Open circles indicate outbreaks during 1919-37. Black circles indicate outbreaks during the current period, some in new localities (full circle), some in old localities (small black dot inside open circle).

Port Elizabeth districts, outbreaks have been sporadic, though frequent enough to outline the enzootic area. Whether the 5-6-year periodicity in frequency of human outbreaks will be maintained now that the population dynamics of the wild rodents throughout the enzootic area have been modified by the establishment of so potent a regulatory factor as plague, is a question for future observation.

The tendency shown by the annual incidence of plague outbreaks in man is for a relatively small number of outbreaks to be spread over three or four years (as in

1940-41-42-43) rather than for them to be concentrated into one or two years (as was the case in 1923-24, 1929-30 and 1934-35-36).

A comparative study of the interaction of such ecological factors as temperature, rainfall, soil, topography, and rodent- and flea-species composition in the plague-infected and plague-free areas—now clearly defined and unlikely to alter—will provide further clues to the problem of the perpetuation of sylvatic plague. This aspect, upon which some information has been collected, will be dealt with in later publications.

SUMMARY

1. An account, illustrated by epidemiological maps, is given of the spread of human plague, derived from a sylvatic source, in the Union of South Africa from 1919 to 1943, together with reference to its spread beyond the borders of the Union.

2. During the period 1919-43 more than 900 outbreaks were reported, all but one of which (an urban outbreak at Port Elizabeth) were in rural areas, the majority of them on farms.

3. The course followed by plague in the wild-rodent populations was reflected in the history of plague outbreaks in man, more faithfully where the risk of infection was highest, as in the northern Orange Free State, and less markedly where the risk of infection was lower, as in the karroo areas of the Cape Province.

4. A periodicity of 5-6 years in the incidence of human plague points to the existence of a general periodicity in the fluctuations in numbers of the wild-rodent populations in the Union as a whole; this shows signs of breaking down as human outbreaks become more and more associated with certain limited hyperenzootic areas (northern Orange Free State, the Glen Grey-St. Marks districts in the eastern Cape Province, and the Uitenhage-Port Elizabeth districts).

5. The enzootic plague area comprises the semi-arid areas of the karroo, the high veld and the Kalahari Desert. It appears to have reached its limits within the Union and in the territories immediately adjoining (Basutoland, South-West Africa and Bechuanaland Protectorate).

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IMPLICATION OF THE MOSQUITO *Aedes* (*Stegomyia*) *Africanus* Theobald IN THE FOREST CYCLE OF YELLOW FEVER IN UGANDA

BY

A. J. HADDOW,
K. C. SMITHBURN,
G. W. A. DICK,
S. F. KITCHEN

AND

W. H. R. LUMSDEN

(From the Yellow Fever Research Institute, Entebbe, Uganda*)

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Since 1937, studies on the epidemiology of yellow fever have been carried on almost continuously by the staff of the Yellow Fever Research Institute in Bwamba county, western Uganda. The study-area consists essentially of a densely populated agricultural zone and a large belt of uninhabited rain-forest, which is continuous with the main Ituri Forest of the Congo Basin.

Investigations were at first confined mainly to the inhabited parts of the county. It was soon established that immunity to yellow fever was present among the African population, that the incidence was greatest in areas adjoining the main forest, and that immunity among children was confined to those localities (Hughes *et al.*, 1941). Subsequent work (Mahaffy *et al.*, 1942) resulted in the isolation of yellow fever virus from a sick African and from mosquitoes belonging to a 'semi-domestic' species (*Aedes* (*Stegomyia*) *simpsoni* Theobald), the favourite habitat of which is banana-plantations.

As a consequence of these discoveries, the entire population of the county was vaccinated against yellow fever, but 11 months after this mass vaccination virus was again isolated from *A. simpsoni* (Smithburn and Haddow, 1946). As it seemed almost certain that the virus in this latter case must have been derived from wild animals, attention was turned to the main forest (Haddow, 1945) in the hope of finding the vector responsible for the transmission of yellow fever virus in forested areas uninhabited by man.

We had established that yellow fever is prevalent and endemic among the monkeys of the Bwamba lowlands, and our survey led us to believe that the incidence of immunity in these animals did not vary greatly from one lowland area to another (Haddow, Smithburn *et al.*, 1947). In view of this finding, and after studying many localities, we finally selected for intensive investigation an area of swamp forest where monkeys were particularly abundant. Catches of forest mosquitoes made there (Mongiro) in 1944 resulted in the isolation of yellow fever virus (Smithburn and Haddow, 1946) from a mixed lot of

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Aedes, belonging to 12 species. Of these 12, only one species (*Aedes (Stegomyia) africanus* Theo.) seemed likely, on theoretical grounds, to be involved in transmission of the virus. Following the isolation of the virus, which took place during the rains in April, work at Mongiro and in the adjacent forest area of Mamirimiri was intensified, and series of catches were made at ground level and at various heights in trees (Haddow, Gillett and Highton, 1947). These yielded a total of 34,588 mosquitoes, including 586 *A. africanus*. Suspensions of all the *A. africanus* and of the majority of the other mosquitoes were inoculated into laboratory animals, but virus was not recovered again that year. The facts were established, however, that *A. africanus* is the dominant culicine mosquito of the forest-canopy in this area, and that it bites most actively just after sunset. These observations were considered to be highly significant, inasmuch as some of the species of monkeys involved in the forest cycle of yellow fever seldom descend to ground level. We considered that there was strong evidence incriminating *A. africanus* as the main vector of yellow fever among the monkeys of this area, and we decided that we might profitably concentrate most of our attention on this mosquito. Before going further, it may be worth while to summarize the points in favour of this view:

1. *A. africanus* has a very wide distribution in central Africa.
2. It is an efficient vector of yellow fever under laboratory conditions, as has been shown by Philip (1929) and by work in the Yellow Fever Research Institute.
3. It was included in the infected lot of *Aedes* spp. taken in 1944.
4. It is the dominant culicine of the forest-canopy in Bwamba.
5. Catches made in trees in many parts of Bwamba (and in the Entebbe area) have shown only three mosquito species common to every tree and every series. *A. africanus* is one of these. One of the others (*A. (S.) apicoargenteus* Theo.) has proved incapable of transmitting virus under laboratory conditions in West Africa (Bauer, 1928) and at Entebbe. The third, *Taeniorhynchus (Mansonioides) africanus* Theo., is known to be capable of transmitting yellow fever virus in the laboratory (Philip, 1930), but was not included in the infected lot of 1944.
6. *A. africanus* has been taken in trees in each of the 17 Uganda localities at an altitude of under 5,000 feet (in addition to Bwamba and Entebbe) which have so far been studied.
7. It bites after dark. This is an important point, as some monkeys, known to be involved in the forest cycle of yellow fever, spend much of their time on the ground by day, but by night (in Bwamba, at least) all species sleep in trees.
8. It survives dry weather in the adult state in forested and wooded areas in Uganda (Haddow, Gillett and Highton, 1947), and is therefore capable of carrying over virus from one wet season to the next.

9. It readily bites monkeys in captivity (Haddow and Mahaffy, *in the press*) and on tree platforms (Haddow and Dick, *in the press*).

We know of no other mosquito, with the possible exception of *T. africanus*, which fulfils all these conditions.

Long series of catches, specially planned to yield the maximum numbers of *A. africanus* (Haddow and Mahaffy, *in the press*), were made during the following year. In these and in other catches conducted in 1945, 23,535 mosquitoes, including 3,628 *A. africanus*, were taken. Suspensions of all the *A. africanus* and of many of the other mosquitoes were inoculated into laboratory animals, but without result.

At this stage we concluded that, although yellow fever virus probably is constantly active in the Bwamba forests, it is very unlikely to be continuously active in any particular locality. It seemed uneconomical to continue large-scale catches without definite evidence of the presence of virus in the area at the time concerned. We therefore began to establish rhesus monkeys as sentinels on platforms in the canopy in many parts of the forest. By the end of the year we had 16 in position. The temperatures of these monkeys were taken daily, and each was bled monthly for protection test. None contracted yellow fever during the year.

During 1946 catches were made at ground level in many localities, and series of 24-hour catches were made on some of the sentinel platforms, with controls at ground level. A total of 54,852 mosquitoes was taken, including 323 *A. africanus*. Suspensions of all the *A. africanus* and of most of the other mosquitoes were inoculated into laboratory animals, but yellow fever virus was not isolated. The sentinel programme was extended to new areas, and by the end of the year 33 monkeys were in position. None of these became immune to yellow fever.

Simultaneously, work was begun at Kitinda and Zika in small belts of forest on the shores of Lake Victoria near Entebbe. In each area a tree was selected and two rhesus sentinels were established in it, one in the canopy and one at the understorey level. Series of 24-hour catches were made on these platforms, with controls at ground level, and these, together with other catches in the Entebbe area, yielded 10,153 mosquitoes, including 1,416 *A. africanus*. Here, also, suspensions of all the *A. africanus* and of most of the other mosquitoes were inoculated into laboratory animals, but yellow fever virus was not isolated.

In 1947 the Kitinda station was discontinued, but five new stations, each for two monkeys, were established in trees at Zika. At the same time the Bwamba sentinel programme was further augmented by adding six new stations, thus bringing the total to 39. In view of the prevalence of yellow fever among wild monkeys both in Bwamba and around Entebbe, and of the long period during which some of the sentinel stations had been maintained, it had been a source of surprise that none of our sentinels contracted the disease. A series of experiments made during the year in Bwamba, however, revealed that *A. africanus* does not readily enter the type of cage which we had used for confining the monkeys. This difficulty was overcome by securing uncaged monkeys on the platforms stationed at Zika. In Bwamba, this work, which has necessitated a considerable amount of rebuilding, is not quite complete at the time of writing. So far, none of the Bwamba sentinels has contracted yellow fever, though we hope for results during 1948.

At Zika, on the other hand, the work was rapidly completed and soon led to the following significant result. Rhesus 778 was bled for a yellow fever protection test on September 13th and was found to be non-immune. From the 13th until the 18th it was confined in mosquito-proof quarters at Entebbe. On the 18th it was stationed at Zika as a sentinel, and remained there until September 30th, when it was bled for the routine monthly protection test. The animal had no record of fever during this period. The protection test, however, showed immunity to yellow fever, and a repeat test confirmed this result. It was concluded that rhesus 778 had experienced a mild infection with yellow fever between September 18th and 30th and that this infection had been contracted in the forest-canopy. Though yellow fever virus was not isolated during October from mosquitoes caught in the area concerned, we were encouraged to believe that, in searching

for yellow fever virus among arboreal species, we were following a line of investigation which might be expected to yield results sooner or later.

During 1947 mosquito catches in the Entebbe area included work on some of the sentinel platforms at Zika, with ground-level controls, together with other ground-level catches at Zika and in various other localities. A total of 12,881 mosquitoes was obtained for inoculation, including 819 *A. africanus*. Yellow fever virus was not isolated. In Bwamba, catches were made at ground level in various localities, and further series of 24-hour catches, with ground-level controls, were carried out on some of the sentinel platforms. In all, 41,168 mosquitoes were taken, including 1,140 *A. africanus*. All the *A. africanus* and many of the other mosquitoes were used for inoculation into laboratory animals. There is evidence that the last batch to be inoculated—a lot of *A. africanus* from Mongiro and Mamirimiri—was infected with yellow fever virus. The circumstances were as follows.

A series of five 24-hour catches was carried out on a 62-ft. platform at Mamirimiri, from December 11th to 16th. The bait consisted of three catchers on the platform and three as controls at ground level. A total of 2,957 mosquitoes was taken, including 139 *A. africanus*. Of the *A. africanus*, three were taken at ground level and 136 on the platform, which was in a dense part of the canopy. A series of six catches was carried out almost simultaneously on a 57-ft. platform (no. 2) at Mongiro from December 9th to 15th. The bait again consisted of three catchers on the platform in the canopy and three at ground level. In this case 3,792 mosquitoes were taken, including 48 *A. africanus*. Four of the *A. africanus* were taken at ground level and 44 on the platform. In addition, one typical *A. (S.) luteocephalus* Newst. was taken on the platform. As we have come to consider *A. luteocephalus* to be merely a subspecies or variety of *A. africanus* (Haddow and Mahaffy, *in the press*), for inoculation this specimen was grouped with the *A. africanus*.

These mosquitoes, comprising one *A. luteocephalus* from the canopy, seven *A. africanus* from ground level, and 180 from the canopy (a total of 188), were pooled for inoculation. They were triturated, without the use of abrasive, in 3 ml. of 10 per cent. normal monkey serum in physiological saline. The suspension was centrifuged at approximately 2,500 revolutions per minute for about 10 minutes. The supernatant fluid (about 1.5 ml.) was then drawn off in a syringe and inoculated subcutaneously into a rhesus monkey, MR 645, on December 16th. This monkey had not been subjected to any previous experimental procedure, except that it had been bled for protection test on December 3rd and had been found non-immune.

On the evening of December 27th the monkey was very quiet. On the morning of the 28th its temperature had fallen to 99.6° F., and it was obviously moribund. A blood specimen was taken from a leg vein. In the afternoon the temperature had fallen further, to 97.2° F., and at 6.30 p.m. the animal died. A further blood specimen was taken from the heart after death, and pieces of liver, kidney and spleen were removed by the African staff of the field laboratory for histological study. No European was in attendance at the autopsy.

Both the ante- and post-mortem blood specimens were found to be protective against yellow fever virus, and the results were confirmed in both cases by repeat tests. The appearance of antibody in the serum and the subsequent death of this animal should not be regarded as an untoward sequence of events, even if death was due to yellow fever.

Berry and Kitchen (1931) found that antibody may be demonstrable in the blood of humans suffering from yellow fever within four days of the onset of the disease and at a time when virus is still present in the circulation. Moreover, in this laboratory we have not only frequently observed the coexistence of virus and specific antibody in experimentally infected monkeys, but have also found the serum to be protective as much as three or four days before the death of the animal.

Throughout the month of December rhesus 645 showed no fever; its highest recorded temperature was only 102.4° F. On account of the absence of pyrexia, yellow fever was not suspected when the animal became sick, and for this reason no subinoculation was made. It is to be noted that in the field laboratory temperatures were, as a rule, taken in the morning only. It is therefore possible that a transient rise of temperature during one evening and/or night may have been missed. Among rhesus monkeys inoculated experimentally with yellow fever virus in the laboratory at Entebbe, infections have been observed in which pyrexia was apparent on a single day only, while in other cases death from typical yellow fever has occurred without any observed rise in temperature.

The pathological material obtained from rhesus 645 by the African staff at the field laboratory consisted of blocks of liver, kidney and spleen tissue. The sections prepared from this material left a good deal to be desired, but we were able to make the following observations.

The spleen showed moderate congestion. Some of the follicles were moderately depleted of lymphoid elements and exhibited numerous large mononuclear cells and some nuclear debris. Other follicles had a fairly normal appearance.

Kidney sections revealed epithelial degeneration in the convoluted tubules, with a considerable amount of fatty change. Many of the tubules contained an amorphous or hyaline deposit. The glomeruli and the collecting tubules showed no abnormality.

Sections of the liver showed wide-spread diffuse necrosis with a definite midzonal preference; the only normal cells present were those adjacent to the central veins and portal spaces. Some, though by no means all, of the necrotic cells were hyperacidophilic, but this characteristic was less pronounced than is commonly the case in animals dying of yellow fever. There was hyperplasia and hypertrophy of the Kupffer cells, some of which contained recognizable debris of hepatic cells. There was also a large amount of fatty degeneration, manifesting itself in both large and fine droplets. Some of the less-damaged hepatic cells exhibited swollen nuclei. In addition, there were numerous binucleated parenchymal cells and considerable numbers of mitotic figures.

While the microscopic lesions in the liver of this animal were not wholly characteristic of yellow fever, they, together with the microscopic findings in the spleen, indicated the presence of an infection; furthermore, the liver parenchyma showed evidence of regeneration. It seems possible, and even probable, that an intercurrent infection played a part in the animal's death, and that earlier there may have been characteristic lesions of yellow fever in the liver, which were healing satisfactorily prior to secondary intervention by an unknown agent.

There can be no doubt that the single injection of mosquitoes to which this monkey was subjected resulted in an infection with yellow fever virus, as the monkey was kept in a screened room in the field laboratory in Bwamba throughout December, and as it showed no immunity to yellow fever when tested at the beginning of the month, prior to the inoculation. It is not clear what part yellow fever played in causing the death of the animal. It

is obvious, however, that yellow fever virus was present in the lot of *A. africanus* inoculated, all of which, it should be noted, were captured in primary forest uninhabited by man. As was stated earlier in this paper, we have long suspected that *A. africanus* is involved as a vector in the forest cycle of yellow fever in central Africa. We consider that the present observations establish this fact conclusively.

SUMMARY

Since 1944 we have suspected that an arboreal mosquito, *Aedes (Stegomyia) africanus* Theobald, is involved in the transmission of yellow fever among monkeys in Uganda. We have, therefore, made numerous mosquito catches in trees since that year, and we have established large numbers of sentinel rhesus monkeys on platforms in trees in our main study-areas, Bwamba county and the forests near Entebbe.

During September, 1947, a sentinel monkey, stationed in the forest-canopy near Entebbe, became immune to yellow fever. This indicated that the vector of the disease among monkeys in this region must be arboreal in its habits.

In December of the same year, a rhesus monkey died 12 days after receiving a single inoculation of a suspension of *A. africanus*, taken in uninhabited forest in Bwamba. A routine test of blood taken before the inoculation had shown the monkey to be non-immune to yellow fever. Blood specimens taken shortly before and immediately after death, on the 12th day following inoculation with the mosquito suspension, contained neutralizing antibody against yellow fever virus. It is concluded that the *A. africanus* were infected with yellow fever virus, and that this species is involved in the forest cycle of yellow fever.

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ACTIVATION OF LATENT KALA-AZAR AND MALARIA BY BATTLE EXPERIENCE

BY

N. L. CORKILL

(From the Department of Tropical Hygiene, Liverpool School of Tropical Medicine)

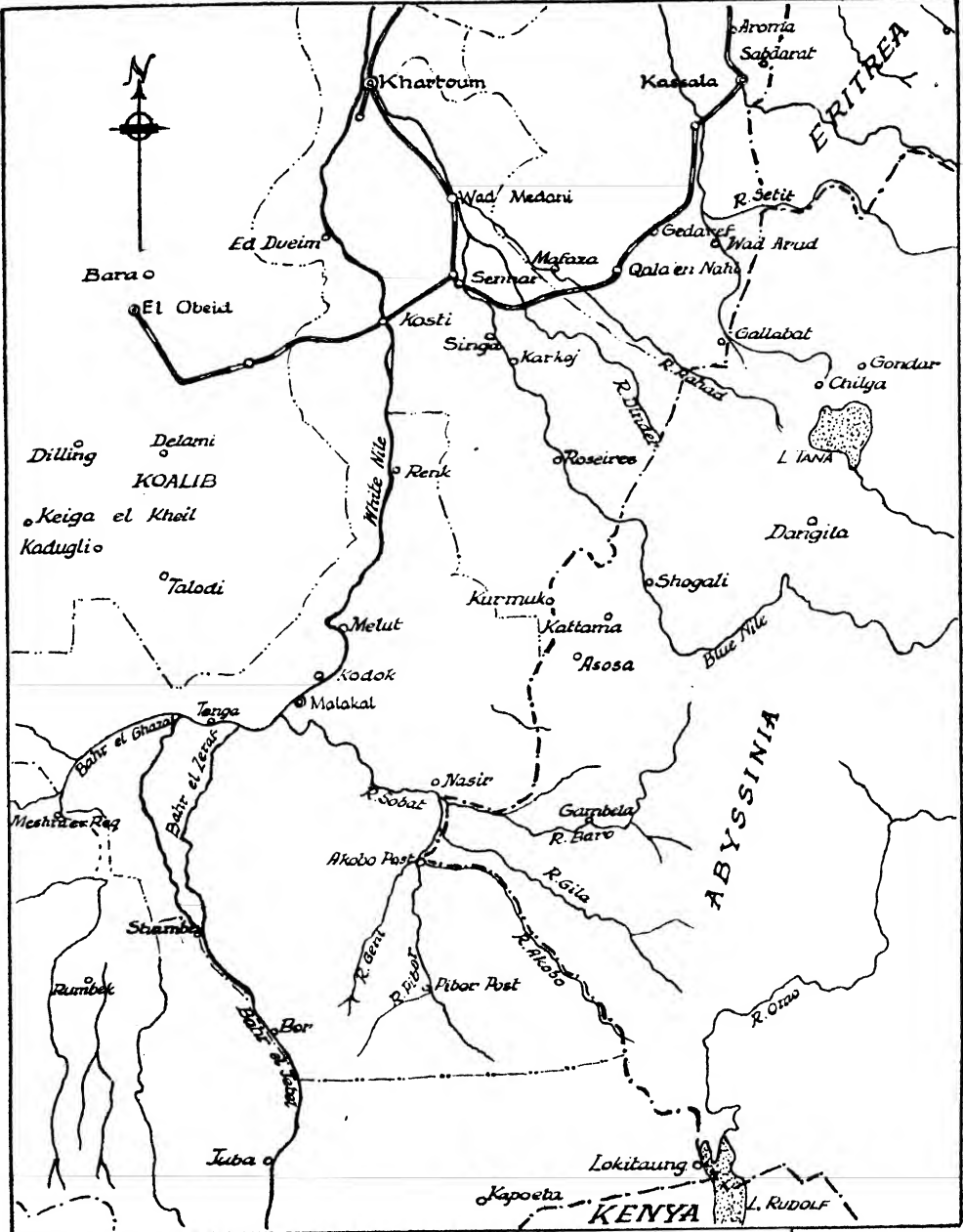
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INTRODUCTION

During the early stages of the recent war, several military units were raised from the Nuba of the Sudan, among which was No. 5 Patrol Company, Sudan Defence Force Frontier Battalion, with which this note is concerned. From the Nuba Mountains of southern Kordofan, Bennett (1935) recorded a case of dermal leishmaniasis in a horse, and Archibald (1914) a case of kala-azar in a woman. Apart from these, infrequent cases of espundia occur, as well as, in more recent times—so the present writer is informed—an occasional case of kala-azar. In seven years' service in the area (1931–38), the writer, who was acquainted with kala-azar and had it always in mind, did not diagnose a single case of the disease, although, in the remote rural localities of Keiga el Kheil and the Koalib, he saw two men whose condition he considered very suggestive of espundia. In short, the occurrence of a case of kala-azar in a Nuba who had not travelled outside the Nuba area would be regarded as unusual and of considerable interest. The Nuba troops under discussion, who were almost without exception men who had never left the Nuba Mountains, were therefore 'green' to the strain of *Leishmania donovani* responsible for the severe form of kala-azar which has made the names of Fung, Gedaref, Gallabat, Kassala and the eastern land frontier notorious in the Sudan. Moreover, linked to this conception of increased susceptibility is the possibility that the disease in such subjects would be less likely to lie dormant, or, to put it otherwise, that it might be more easily activated from latency.

THE COMPANY'S FIRST CASE OF KALA-AZAR

The unit was raised in Dilling in June, 1940, and in November moved to the neighbourhood of Roseires in the southern Fung, an area heavily endemic for kala-azar. A Nuba (B.S.) of the advance party left Dilling on October 10th, arrived in Sennar on the 13th, and was admitted to the hospital there on the 14th, the condition for which he was admitted (that is, the tentative diagnosis) not being recorded. The records do show, however, that he died in the hospital from kala-azar on January 26th, 1941, 104 days later. Certain points suggest that he may have contracted his infection in the ward of Sennar hospital, where at the time kala-azar cases were neither screened nor nursed apart from other cases. In November and December, 1940, 16 cases of the disease were admitted to the Sennar wards. Cases of kala-azar have occurred in young inhabitants of Sennar who have never left the locality, which shows that infection may occur locally; and Archibald and Mansour (1937) obtained sandflies in the wards of Singa hospital, some miles further south, thus suggesting that ward infection is possible, so far as the presence of the vector is concerned. It therefore seems quite possible that this Nuba was infected with kala-azar while in hospital—it may be because of lowered resistance due to the



SKETCH MAP OF SOUTH EASTERN SUDAN

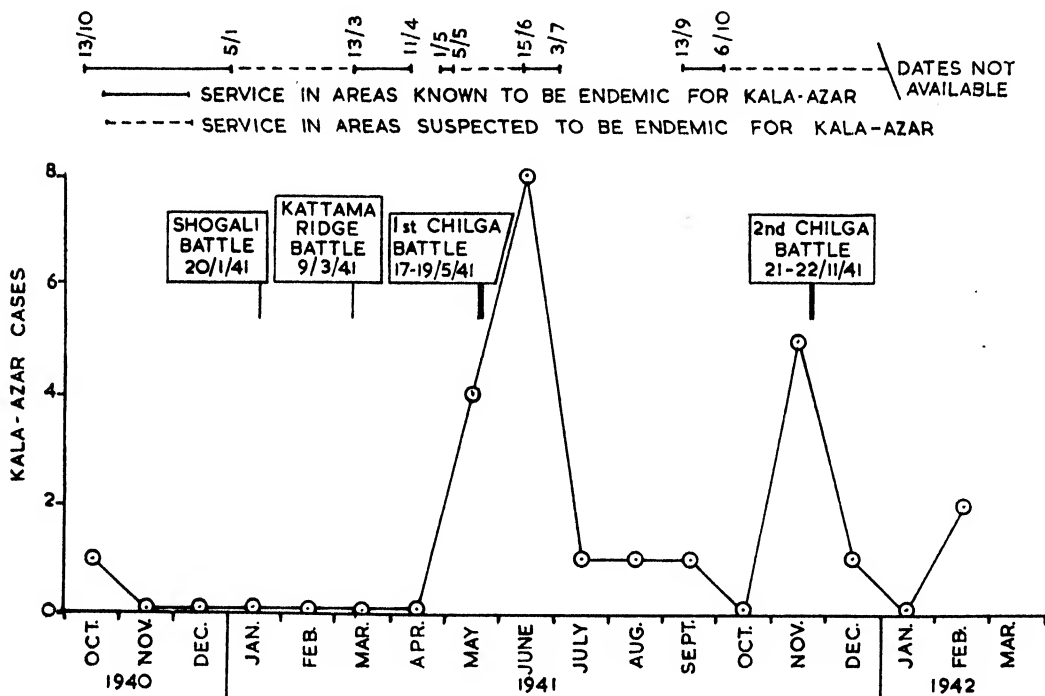
SCALE OF MILES (APPROX)

0 100 200 300

unspecified condition for which he was originally admitted. Unfortunately there is no record of his previous movements, if any, outside the Nuba Mountains, and it may be that he had been infected with the severe strain of *L. donovani* while travelling, before enlistment, in an endemic area elsewhere in the Sudan. It is worth noting, however, that the 2-4 months' incubation-period considered normal for the average kala-azar case is compatible with the thesis that this man developed his disease in Sennar.

OUTBREAKS OF KALA-AZAR FOLLOWING BATTLE EXPERIENCE

The company was soon on active service in the southern Fung and Abyssinia, in areas either known to be endemic for severe kala-azar or very strongly suspected of being so. During that time they marched considerable distances and fought two battles. They then went on leave to their homes in Kordofan, returning in May, 1941, to Gedaref, another area notoriously endemic for kala-azar. Up to that time there had been no cases of kala-azar amongst them, other than the one discussed above.



GRAPH 1. The incidence of kala-azar in a company of Nuba troops.

The company then crossed the frontier for an attack on Chilga in Abyssinia. The altitude was of the order of 6,000 ft., the early rains had commenced, the surroundings were cold and wet, movement was arduous, and the action, fought on May 17th-19th, was a severe battle experience. Among the casualties evacuated were a number of cases of fever, which on arrival at Gedaref hospital several days later were shown to be cases of kala-azar. The outbreak had no relation in time to the normal seasonal peak for the disease in Gedaref, the nearest place for which figures are available.

The outbreak naturally occasioned comment from both medical and regimental staff, and, with the obvious analogy of malaria in mind, was tentatively ascribed to activation of a latent kala-azar infection acquired by the Nuba after their arrival in areas endemic for kala-azar, and more easily activated in them by cold and fatigue since they were 'green' to the disease. The unit then moved back, first to the endemic Gallabat-Gedaref area and later to Kordofan, again for leave. They returned to Gedaref in September for a further assault on Chilga, which took place on November 2nd. The circumstances of this second battle were, from the physiological point of view, comparable with those of the first, except that the weather was colder. Again the casualties evacuated included cases of fever which proved to be kala-azar. The second outbreak coincided in time with the expected seasonal peak of kala-azar in Gedaref, Singa and Sennar, though whether any such peaks did in fact occur in those places in 1941 is not known.

That both outbreaks occurred subsequent to battle experience—there being no cases immediately before the battles—suggests that battle itself, rather than season or other factors, was primarily responsible, though cold, fatigue and the chill due to wetting may have been contributory. The mechanism which suggests itself is increased adrenalin output owing to emotional stress, though incidentally it may be noted that cold also increases adrenalin output. There appear to be only two alternative explanations conceivable, and in the circumstances neither is acceptable. The first is that these cases were developed clinical cases of kala-azar before the battles, but that for some reason they came to notice only after the battles had occurred. While allowing the fullest credit for stoicism and military pride, however, it is quite out of the question that before the battle these cases were in the same clinical state as they were immediately following it. It would have been physically impossible for the men to have carried out their duties, and their condition would have been noticed by the officers and N.C.O.s, as well as by the medical orderly with each platoon. The second alternative, which is equally unacceptable, is that, on two occasions, these cases were all infected on such dates that, by chance, all developed overt disease, not, be it noted, round about the date of battle, but on the actual day of battle or on the day following. This demands too much of coincidence.

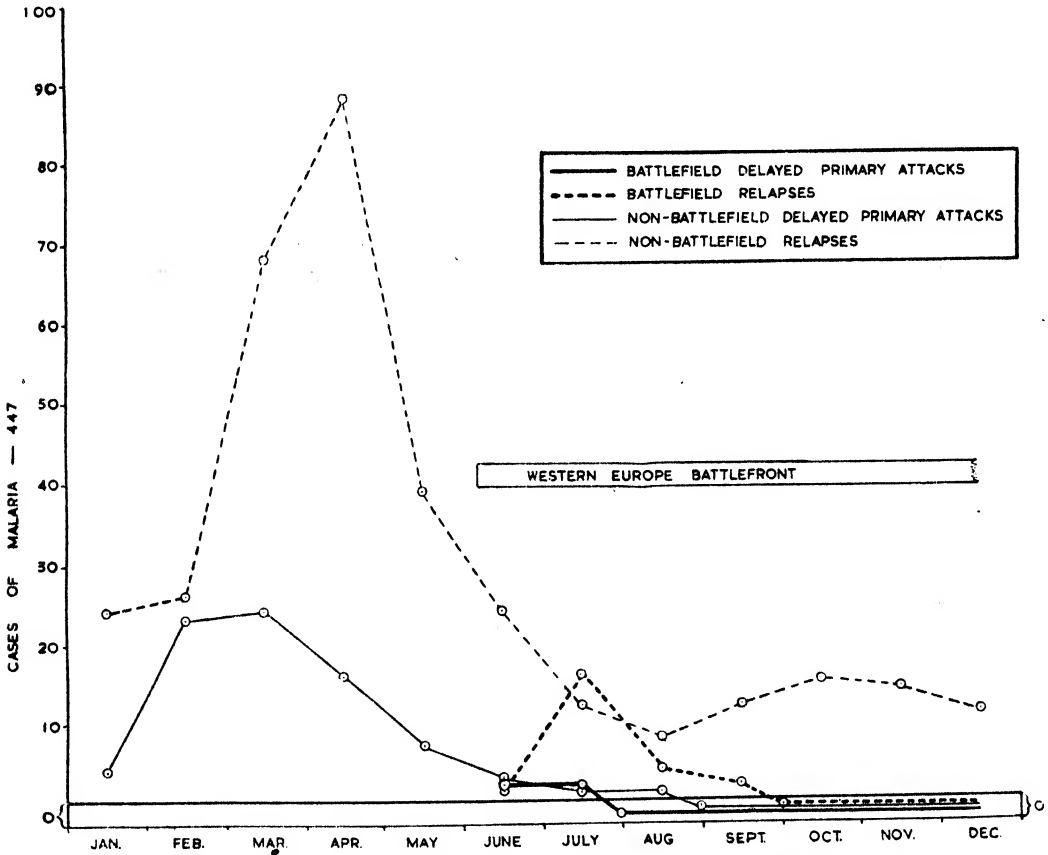
THE ACTIVATION OF LATENT MALARIA BY BATTLE EXPERIENCE

It has been suggested to the writer that a parallel to these outbreaks of kala-azar was that of the exceptional incidence of delayed primary attacks and relapses of malaria in British troops on the Normandy battle-front in the summer of 1944. Many of these troops had previously served in malarious theatres of war and had been on suppressive drugs. A search of the 1944 case-sheets of British troops admitted to the tropical wards of the Liverpool hospitals under the care of Dr. A. R. D. Adams gave the material from which graph 2 is constructed. Two classes of case were sought for: (a) cases of malaria evacuated from the European battle-front, and (b) cases of malaria from the troops remaining in England, which constitute a control, in that they did not develop their attacks under battlefield conditions.

Graph 2 clearly shows the relation of battle experience to delayed primary attack and relapse, in contrast to the absence of a similar peak in the control series in the summer months of June and July. The control series shows the spring peak, which is characteristic of Netherlands malaria (Swellengrebel and de Buck, 1938; Gill, 1938), and the autumn and winter minor wave, which is less constant in temperate-climate malaria. The important

point, however, is the absence of a peak in July, at the time when the battlefield was producing its highest incidence in this Liverpool sample.

It will be noted that after August no further cases are shown as occurring in the battle area, although there is an autumn wave of relapses in the non-battlefield control series. Hence it would seem that battle may have precipitated potential relapse in the summer in cases which would otherwise have relapsed in due course in the normal season in the following autumn. The writer (Corkill, 1948) has described a parallel phenomenon in the Sudan, in which this purging effect was exercised by an epidemic of relapsing



GRAPH 2. Malaria cases in British troops hospitalized in Liverpool in 1944.

fever, which apparently activated latent kala-azar which would otherwise have formed the bulk of the cases normally occurring as a seasonal wave in the latter part of the year. No doubt other cases of malaria did occur in France in the autumn, but their absence from the random sample shown in graph 2 suggests that they were negligible in number, and that therefore the generalization made is justifiable, viz., that battle experience, by activating potential cases of malaria relapse, aborted the normal seasonal peak of such relapses due in the autumn.

DISCUSSION

The question naturally arises of why the earlier battles at Shogali and Kattama in January and March respectively did not occasion break-downs in host-parasite equilibrium. It seems to be generally accepted that, although there may be many exceptions, the average case of kala-azar takes from two to six months to develop from infection to overt clinical manifestation; and it might be argued that these Nuba were not ripe for activation at the times of the earlier battles, which occurred at intervals of three and five months respectively from the time of the company's first entry into areas endemic for severe kala-azar. Further, these two earlier battles were less severe experiences, both clinically and as battles, than the Chilga attacks.

SUMMARY AND CONCLUSIONS

1. A company of African troops, believed to be 'green' to the strains of *Leishmania donovani* responsible for the severe kala-azar of the eastern Sudan, was raised in an area of extremely low endemicity for leishmanial disease and was engaged in military operations on the Sudan-Abyssinian frontier, in areas either known to be or strongly suspected of being endemic for kala-azar of this severe type.

2. Following two severe experiences in battle, evacuated casualties included pyrexial cases subsequently found to be typical clinical examples of kala-azar.

3. A parallel was provided by the incidence of cases of delayed primary attacks and relapses of malaria on the Normandy battle-front in July, 1944, when there was no comparable incidence in the troops remaining in England.

4. It is considered that the break-down of host-parasite equilibrium in these latent infections was occasioned by one or more of the following factors: emotional stress, cold, and high energy-output—possibly in combination but certainly with the first factor dominating, since the other conditions were present at other times without being associated with anomalous outbreaks.

5. It is concluded that battle experience, probably through increased adrenalin secretion due to emotional stress, may activate kala-azar and malaria from latency.

6. It appears that factors which activate malaria and kala-azar from latency, if applied on a communal scale at a point in time earlier than that of the normal seasonal wave, may precipitate that wave and so render its nature at the normal season abortive or minimal.

ACKNOWLEDGEMENTS.—Acknowledgements are made to Dr. A. R. D. Adams for the use of material relating to hospitalized cases of malaria; to Kaimakam R. M. Buchanan Bey for certain figures respecting kala-azar from No. 1 Sudan Defence Force Base Hospital; and, with particular gratitude, to Bimb. T. Guyatt for data relating to troop movements and cases of kala-azar from the regimental files.

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ACTIVATION OF LATENT KALA-AZAR BY MALARIA AND RELAPSING FEVER

BY

N. L. CORKILL

(From the Department of Tropical Hygiene, Liverpool School of Tropical Medicine)

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INTRODUCTION

A growing feeling that the clinical association of kala-azar with other infections was not always a matter of chance led the present writer in 1940 to construct graphs in the Sudan for the incidence of kala-azar and malaria, as reflected in local hospital admissions. The figures were plotted for three successive years in three kala-azar areas. Eight of the nine pairs of curves obtained were compatible with a constant association, but the ninth kala-azar curve was so anomalous that a re-examination was made of the morbidity returns for the area and period concerned. This revealed that the anomalous kala-azar curve was coincident with that of an epidemic of relapsing fever.

PREVIOUS OBSERVATIONS ON THE SEASONAL ASSOCIATION OF KALA-AZAR WITH MALARIA

Napier and Krishnan (1931) pointed out that the morbidity curve for malaria in India, as published by Knowles and Senior White (1930), and that for kala-azar, as published by Napier (1927), rise together, but that the curve for kala-azar is sustained when that for malaria falls, thus suggesting that malaria activates or in some way 'causes' kala-azar. Further, they quoted an instance of co-existent epidemics of both diseases in the same village, and stated that in India all kala-azar areas are malarious. They recorded, however, the existence of malaria cases with transient leishmanial infections, thus showing that the presence of the malarial parasite is not always sufficient to secure the development of clinical kala-azar in a subject infected with *L. donovani*.

Smith and Ahmed (1941) in Bihar wrote of the 'close correlation in the distribution of malaria and kala-azar in various parts of this district, where localized epidemics of malaria have been followed by considerable increases in the incidence of kala-azar.' They found no significant relationship to the periodic prevalence of sandflies in houses, and suggested that a common factor was responsible for a coincident flare-up of both diseases.

THE SEASONAL CURVE FOR KALA-AZAR IN THE SUDAN

In the Sudan all kala-azar areas are malarious, but all malarious areas are not endemic for kala-azar. This point appears, at present, to be of administrative interest only. Archibald and Mansour (1937), correlating the geographical distribution of the disease with its minimum rainfall requirements, inferred that the seasonal incidence of the disease—i.e., presumably, the act of effective inoculation—occurred from July to October. Henderson (1937), writing of the diseases in the Fung province, states that 'Cases probably originated mostly between August and February.' Kirk (1939) writes that, for two years in Singa, the preponderance of cases presented themselves for treatment during the period October-

February, and points out that, assuming an average incubation-period of 3-6 months, the risk of infection is thus greatest in the period July-October. Hence there has to be borne in mind the possibility of a chance association of the curves for malaria and kala-azar because of the 3-6 months' lag in the development of the latter disease due to the long incubation-period.

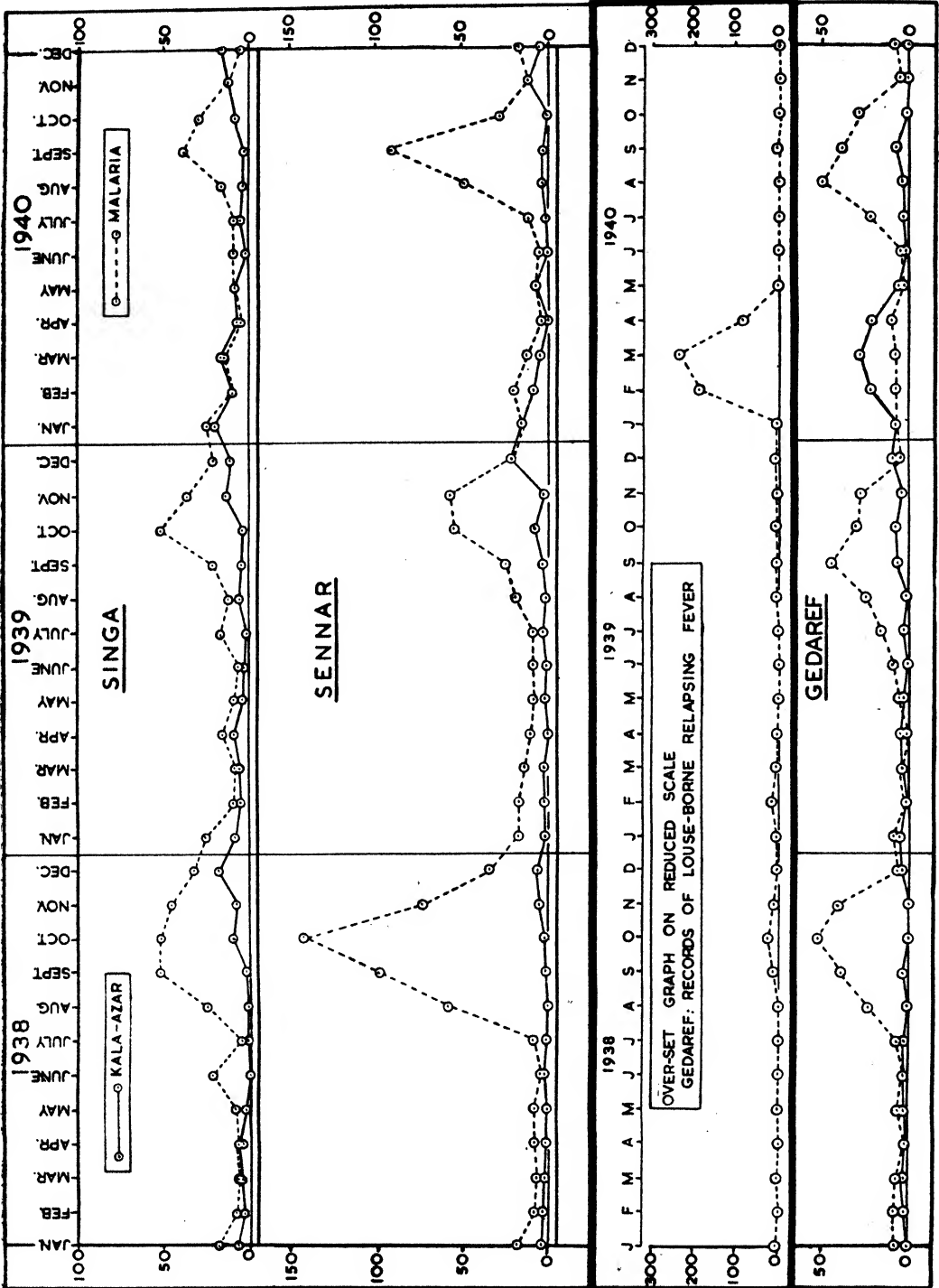
The accompanying graph shows that, except for two anomalies discussed below, in the years 1938-40 in Sennar, Singa and Gedaref—three towns in areas well known as endemic for kala-azar—the annual wave of admission to hospital for this disease rises as that for malaria declines, and is sustained after the latter's fall. This is a parallel to the situation already referred to in India, and in the absence of any convincing alternative most readily suggests activation of latent kala-azar by malaria. The figures are those of admissions to hospital and are not necessarily significant of the onset of fever. They thus probably represent the conviction of patients that they were more than merely indisposed. The wave of admissions covers the months October to February and is compatible with the observations of the writers just quoted.

ACTIVATION BY RELAPSING FEVER

The striking anomaly of the spring peak of kala-azar in Gedaref in 1940 is seen to coincide with that of a local epidemic of louse-borne relapsing fever (numbers admitted to hospital; figures for those admitted to field quarantines are not available). The second and equally striking anomaly of the aborted peak for kala-azar in the normal winter season later in the same year (1940) may be explained by assuming that the cases of kala-azar, which in the absence of relapsing fever would have been activated by malaria in due season later to form this winter peak, were in fact activated earlier in the year by the relapsing fever.

Support for the thesis of activation of the latent disease is provided by a consideration of the accompanying table. This shows, for the Gedaref area in 1940, the numbers of cases diagnosed as (a) kala-azar, (b) both kala-azar and relapsing fever, and (c) relapsing fever. They do not represent all such cases, but are the total of those of which the desired details were available and which could be collected from surviving records. They thus constitute a random sample. The cases are shown in relation to the localities in the Gedaref area from which they came. Thirteen cases were in fact diagnosed as suffering from both infections. Further, it is seen that, in localities of bad reputation in respect of kala-azar and in which relapsing fever occurred, there were more cases of kala-azar than in localities of bad reputation unvisited by relapsing fever. Much cannot be made of this, however, as the numbers and distribution of the population for the area, which should have considerable bearing on any inferences attempted, are not available. None the less, the point is of sufficient interest to be noted.

Taking into consideration the increase in cases of kala-azar in 1940 as compared with the two previous years, the existence of the epidemic of relapsing fever, and the occurrence of a considerable number of cases diagnosed as suffering from both diseases, it seems reasonable to view the double clinical association either as activation from latency in those already infected with kala-azar, or as an increased susceptibility to infection with kala-azar and its manifestation in a fulminating form in cases of relapsing fever as contrasted with other members of the community. If, however, it is postulated that the normal kala-azar season is due to a four months' lag following infection by sandflies at their seasonal peak



GRAPH. Activation of kala-azar by malaria and relapsing fever.

of either prevalence or infective biting efficiency, or both, then this latter alternative seems untenable, as the wave of kala-azar did not occur in the normal kala-azar season, and it therefore seems more reasonable to explain this anomalous wave as being due to activation by relapsing fever.

TABLE

Kala-azar in the Gedaref area of the Sudan in 1940: morbidity relative to that of relapsing fever in kala-azar foci of bad reputation*

Locality†	Reputation in respect of kala-azar	Hospital admissions‡ diagnosed as			Total kala-azar cases
		Relapsing fever	Both diseases	Kala-azar	
Gedaref§ ...	Bad	14	—	19	19
El Hamra ...	"	—	1	8	9
Kassab ...	"	1	2	1	3
Wad Dhaif ...	"	1	—	10	10
Zareiga ...	"	—	—	2	2
Qala el Nahal ...	"	—	—	4	4
Hawata ...	"	—	—	3	3
Beila ...	"	—	—	1	1
Gallabat ...	"	—	—	2	2
Abaiyo ...	Fairly bad	324	5	1	6
Sherif Said ...	"	—	—	1	1
Asar ...	Very little	72	—	—	—
Ambassa ...	"	22	2	5	7
Kanz ...	"	18	—	—	—
Goreisha ...	"	1	—	1	1
Um Shidera ...	"	10	1	—	1
Shesheina ...	"	1	2	—	2
Hillet el Malik ...	"	5	—	1	1
Kom Shittar ...	"	—	—	3	3
Hillet Gerrar ...	"	—	—	1	1
Agab Sidu ...	"	—	—	1	1
Wad el Hilawa ...	"	—	—	1	1
Wad Kabo ...	"	—	—	1	1
Tomaat ...	Free	1	—	1	1
Sofi ...	"	2	—	—	—
Kereida ...	"	2	—	—	—
Um Kunjur ...	"	1	—	—	—
Komar Asar ...	"	1	—	—	—
El Jubarab ...	"	1	—	—	—
Kassara ...	"	—	—	1	1
Abuda ...	"	—	—	1	1
Abu Furo ...	"	—	—	1	1
Heshaib ...	"	—	—	1	1
Muqata ...	"	—	—	1	1
Totals ...		477	13	72	85

* Population figures would be of interest, but they are not available.

† Massed for the most part into an area some 15 miles square south-west of Gedaref.

‡ Excluded are cases deriving from outside the area and those for which complete data are not available.

§ Grouped with Gedaref are its suburbs, Deim Bakr and Sofi el Azraq. Together they include a large proportion of the population of the area.

|| Totals for 1938 and 1939 were 17 and 47 respectively. In these years there were no local epidemics of relapsing fever.

ACTIVATION BY DISEASES OTHER THAN MALARIA AND RELAPSING FEVER

There is, of course, nothing novel about this phenomenon of activation. Malaria relapses are known to be caused by attacks of other diseases, and kala-azar—a comparable

protozoal disease—has, in India, been associated with enteric, in addition to its clinical association with malaria. Napier and Krishnan (1931) quoted a resident medical officer of a hospital in India as saying that 'about 75 per cent. of the enteric patients from one particular area—the kala-azar-endemic area in Calcutta—develop kala-azar in hospital or return within a month or so with the disease.' In the Fung area of the Sudan, Henderson (1937) found concomitant infections with other diseases in nearly half of his series of 300 cases of kala-azar.

DISCUSSION

It has thus been shown that in India and the Sudan the wave of kala-azar rises after that of malaria and is sustained after its fall. Explanations offered have been (a) activation of latent kala-azar by an attack of malaria, (b) chance co-incidence (in the Sudan) of the malaria season with that of the development of overt kala-azar due to a four months' lag in the average case after the season of maximum infectivity or frequency, or both, of the sandfly vector, and (c) (in India) that a common factor activates both diseases at the same time.

The clinical association of kala-azar with typhoid (a pollution disease) endemic in India and with relapsing fever (a personal-contact disease) epidemic in the Sudan—two infections of entirely different epidemiology—suggests that there is no need to seek in either country for an explanation of the association with malaria—a disease of yet another epidemiological type—apart from that of activation of latent kala-azar by the advent of malaria. It may be that the chance co-incidence in the Sudan of, say, a four months' lag between the sandfly season and that of the incidence of overt kala-azar contributes to the situation, in that the bulk of kala-azar infections are ripe, as it were, for activation at this season, and it may be also that nature turns the scales against the potential kala-azar case still further by allowing these two factors—malaria season and sandfly season—to produce their effects in conjunction with a third factor—the season of maximum cold, December-February. Cold is a factor known to produce a break-down of the host-parasite equilibrium in malaria, and that it may act similarly in kala-azar has to be considered possible, if not probable.

SUMMARY AND CONCLUSIONS

1. In India and the Anglo-Egyptian Sudan the morbidity curves for malaria and kala-azar rise in association, but that for kala-azar is sustained after the fall of that for malaria. In the Sudan this held good for eight observations out of nine.
2. In the ninth observation the kala-azar wave coincided with a wave of epidemic louse-borne relapsing fever at a season other than that in which the malaria and kala-azar waves were usually associated.
3. This anomaly appeared to be related to the further anomaly of the absence of the usual kala-azar wave in association with the malaria wave later in the year, in that it may be considered that the epidemic of relapsing fever activated the latent infections of kala-azar which, in the absence of this epidemic, would have been activated by malaria in due season later in the year in the normal way.
4. The activation of kala-azar from latency by malaria, enteric and louse-borne relapsing fever appears to be indisputable.
5. The occurrence in the Sudan of a period of maximum vector (sandfly) prevalence or infectivity, or both, some four months before the malaria season may contribute to the

wave-association of the two diseases, as it appears that the average case of kala-azar takes four months or so to become overt.

6. That the malaria season and the kala-azar season together are associated with the annual period of low temperature suggests that cold may be a third factor tending to cause kala-azar cases to accumulate at this season; it is probable that cold *per se* may conduce to a break-down of host-parasite equilibrium in kala-azar as in malaria.

7. As the average case of kala-azar has an incubation-period of something like four months, there seem to be no grounds for considering that the clinical association of malaria and kala-azar has any relationship to a joint prevalence of the insect vectors due to the existence of meteorological conditions favourable to mosquito and sandfly.

8. It is concluded that malaria, enteric and relapsing fever may activate kala-azar from latency into manifest clinical form, and that probably other diseases will do likewise.

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HAEMOGLOBIN STAINS ADAPTED FOR USE WITH THIN BLOOD FILMS

BY

ROBERT H. BLACK*

(From the Department of Tropical Medicine, Liverpool School of Tropical Medicine)
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Various staining methods have been described for the identification of haemoglobin in tissue sections. When they are used to stain thin blood films, some modifications of the techniques are necessary, and the present paper describes several methods which have been evolved and the results which have been obtained with them.

METHODS

1. *Cyanol*

The details of the cyanol method have been given in a previous paper (Black, 1947). Briefly, the method depends on the resumption of colour by the reduced colourless form of cyanol in the presence of haemoglobin. The haemoglobin in the red cells is stained cobalt green, and either safranin or Leishman may be used as a counterstain. When Leishman is used, the previous exposure to the cyanol in some manner prevents the usual staining action of Leishman of the cytoplasm of red cells, and the cytoplasm remains the same colour as before counterstaining.

If used with care the method is satisfactory, but there is a tendency for the cyanol to wash out of the red cells if the reagents are too acid.

2. *Alizarin Red S and Phosphomolybdic Acid*

The method for staining haemoglobin with alizarin red S and phosphomolybdic acid was described for tissue sections by Okajima (1916). Preliminary staining with haematoxylin is followed by a brief exposure to phosphomolybdic acid and then by prolonged staining in a moist chamber with a mixture of phosphomolybdic acid and alizarin red S.

Thin blood films are fixed with methyl alcohol and then stained with Ehrlich's acid haematoxylin for 15 minutes. The haematoxylin is washed off with water, and the slides are then placed in 10 per cent. aqueous phosphomolybdic acid for $1\frac{1}{2}$ minutes. Another wash with water is followed by staining for 18 hours with a mixture of 50 ml. of a saturated aqueous solution of alizarin red S and 10 ml. of 10 per cent. aqueous phosphomolybdic acid. The slide is then washed with water and dried. The haemoglobin of the red cells is stained a bright yellow, and the nuclei (as in avian blood) are well defined and dark purple in colour.

3. *Reduced Fuchsin and Methyl Green*

The reduced-fuchsin methyl-green method, which was derived from one described by Lison (1931) for the staining of haemoglobin in tissue sections, gives a better contrast between the nuclei and the cytoplasm of the red cells of avian blood than any yet studied.

* Working under a grant from the Medical Research Council.

Haemoglobin causes the reduced leuco- form of fuchsin to regain its colour, and the haemoglobin of the red cells becomes stained with fuchsin.

The stock solution of the leuco-fuchsin is prepared by mixing 1.5 gm. of acid fuchsin, 5 gm. of powdered zinc, 2 ml. of glacial acetic acid and 100 ml. of distilled water. The mixture is boiled and soon loses its colour. When cool, a further 2 ml. of glacial acetic acid is added. To prepare the working solution, 10 ml. is filtered off, and to this 1 ml. of commercial hydrogen peroxide is added.

Thin blood films are fixed with methyl alcohol and dried. The working solution is then run on to the film and allowed to act until the full intensity of colour has developed; this takes about three minutes. The stain is then tipped off and the slide is counter-stained with 0.5 per cent. aqueous methyl green for one minute, after which the slide is washed for a brief period with water and blotted dry.

The haemoglobin of normal mature red cells is stained with the fuchsin. In avian blood the nuclei of red cells are stained green.

RESULTS AND COMMENT

The specificity of these methods for the staining of haemoglobin has been discussed elsewhere (Black, 1948), when a series of staining methods was used for the identification of haemoglobin in tissue sections.

The techniques described above have proved to be valuable in various haematological and parasitological studies, some of which may be mentioned in outline.

The cyanol-staining method has been used for a study of the consumption of haemoglobin by malarial parasites (Black, 1947).

The second method, using alizarin red S, has been used to observe the acquisition of haemoglobin during the maturation of red cells in ducklings infected with *P. cathemerium*.

The leuco-fuchsin technique has been used to observe the staining reactions of the parasites of avian malaria and the changes in haemoglobin content of chick erythrocytes during their maturation. In chick blood containing malaria parasites, e.g., *P. gallinaceum*, the parasites are seen as rounded pale-green areas in the cytoplasm, and the pigment is readily discernible. In a chick heavily infected with *P. gallinaceum* a series of colour-changes is seen in the cytoplasm of the red cells, the changes depending on the degree of maturation and the acquisition of haemoglobin. In the erythroblasts, some of which show mitotic figures, the cytoplasm stains green. With further maturation this green staining of the cytoplasm is lost, and an increasing amount of fuchsin appears in the cytoplasm as the nucleus becomes smaller, more oval in shape and more compact. It is only in those cells in which the cytoplasm stains with fuchsin that the parasites (*P. gallinaceum*) are found.

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A NOMOGRAM FOR THE PREPARATION OF STANDARD INOCULA IN THE DAVEY CHICK TEST OF ANTIMALARIAL ACTIVITY

BY

J. WILLIAMSON*

(From the Warrington Yorke Department of Chemotherapy,
Liverpool School of Tropical Medicine)

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The preparation of inocula of fowl blood parasitized with *Plasmodium gallinaceum*, in dilutions containing the standard number of infected erythrocytes ($50 \times 10^6/0.2$ c.cm.) for intravenous inoculation into six-day-old chicks (Davey, 1946), may be simplified by the use of the accompanying nomogram. Where a = the number of red blood-corpuscles in millions per c.mm., and b = the number of parasitized red blood-corpuscles per 500 red blood-corpuscles in the infected blood from which the dilution is to be made, the dilution c , required to give a parasitized R.B.C. density of $50 \times 10^6/0.2$ c.cm., can be obtained from the relation $ab = 125 c$.

Where large numbers of experiments are carried out with the Davey technique, it has been found convenient to use this simplified relation as the basis for the construction of a nomogram of the third class (Allcock and Jones, 1941), employing a logarithmic transformation to give three parallel straight lines. In use, a straight edge is laid across the appropriate values of a and b , marked on the two outer scales, and the corresponding point of intersection on the middle scale gives the required dilution factor directly.

The limiting values of the quantities a and b were taken as 0.5–4.0 millions/c.mm. and 100–500 parasitized R.B.C./500 R.B.C. respectively, which determined the limits of c as 0.4–16.0. Using the method described by Allcock and Jones (1941) for the construction of nomograms of the third class, genus zero, where the logarithmic relation

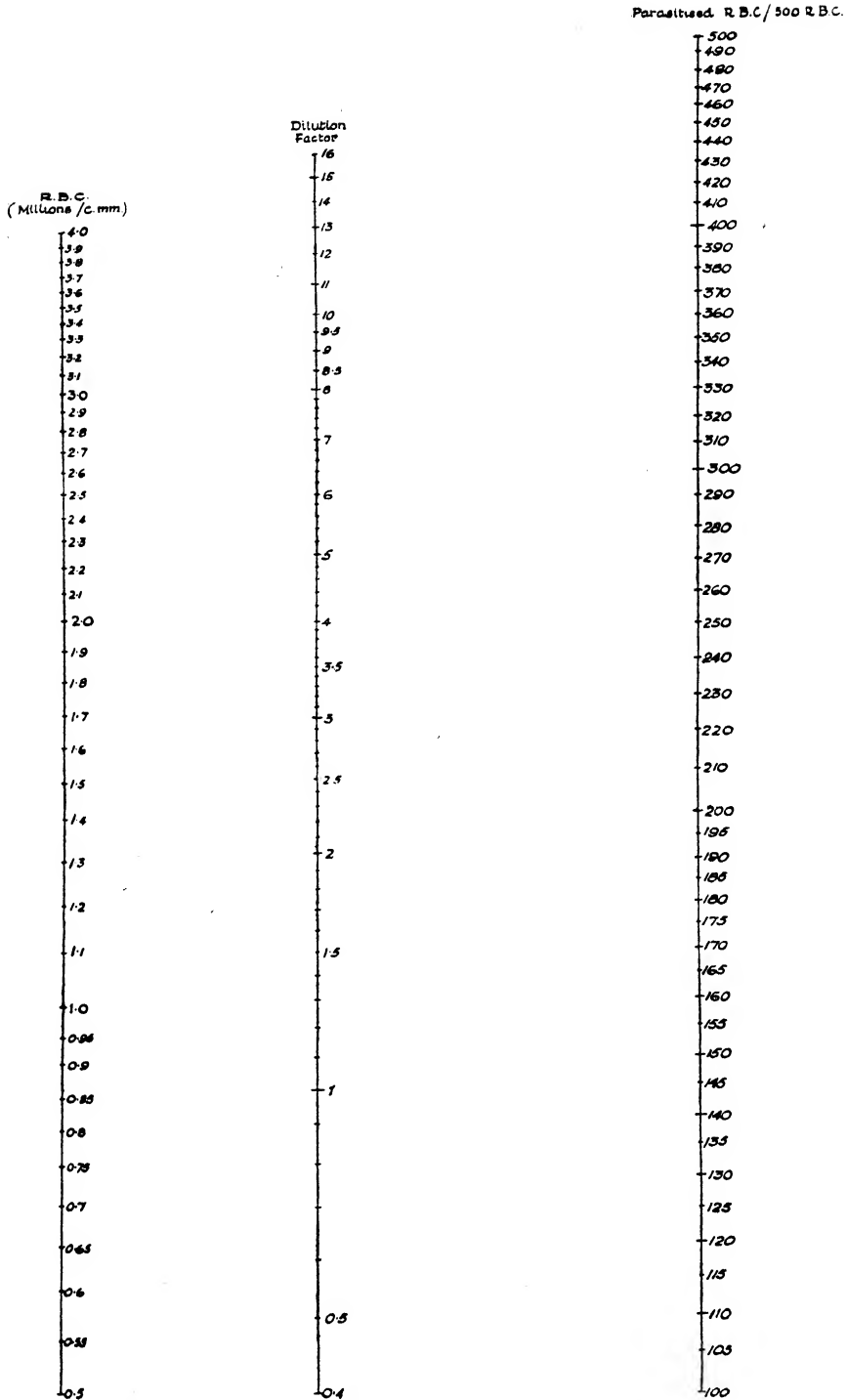
$$\log a - (\log c + \log 125) + \log b = 0$$

corresponds to the standard formula $f(u) + f(v) + f(w) = 0$, the appropriate constructional determinant was calculated for a nomogram approximately 40 cm. \times 40 cm., i.e., the determinant

$$\begin{vmatrix} -\delta_1 & \mu_1 \cdot f(u) & 1 \\ 0 & -\frac{\mu_1 \cdot \mu_3}{\mu_1 + \mu_3} \cdot f(v) & 1 \\ \delta_3 & \mu_3 \cdot f(w) & 1 \end{vmatrix} = 0 \quad \begin{matrix} \text{(where } \delta_1, \delta_3, \mu_1 \text{ and } \mu_3 \\ \text{are scale factors such that} \\ \delta_1 \mu_3 = \delta_3 \mu_1 \text{), on substitution became} \end{matrix}$$

$$\begin{vmatrix} -80 & 400 \log a & 1 \\ 0 & 240 (\log c + \log 125) & 1 \\ 120 & 600 \log b & 1 \end{vmatrix} = 0.$$

* May and Baker Research Fellow.



Nomogram for standard Davey chick-test inocula.

Small graduations were obtained by projection from standard logarithmic scales, and the completed nomogram (see accompanying diagram) was mounted, after photographic reduction to a convenient size, on a wooden backing with a slot at one side for the attachment of a sliding pivot holding a strip of Perspex scribed on the under side for use as a transparent straight edge.

SUMMARY

A simply constructed nomogram is described and reproduced for the rapid determination of dilution factors in the preparation of standard inocula for the Davey chick test.

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ACQUIRED PALUDRINE-RESISTANCE IN *PLASMODIUM GALLINACEUM*

II.—FAILURE TO PRODUCE SUCH RESISTANCE BY PROLONGED TREATMENT OF LATENT INFECTIONS

BY

I. M. ROLLO,*

J. WILLIAMSON†

AND

E. M. LOURIE

(From the Warrington Yorke Department of Chemotherapy,
Liverpool School of Tropical Medicine)

(Received for publication August 4th, 1948)

INTRODUCTION

A high degree of resistance to paludrine may be acquired by *Plasmodium gallinaceum* as a result of subcurative treatment of a relatively short series of syringe-passaged infections in chicks (Bishop and Birkett, 1947 ; Williamson, Bertram and Lourie, 1947 ; Williamson and Lourie, 1947). In our experiments the resistant character was well established in $2\frac{1}{2}$ – $3\frac{1}{2}$ months of treatment, whether this was administered in maximum-tolerated or in relatively low dosages. The facility with which resistance was acquired in respect of this particular compound stood in marked contrast to its failure to develop in respect of certain other antimalarial agents, in spite of prolonged and intensive treatment with those substances. This was continued for as long as $2\frac{1}{4}$ – $2\frac{1}{2}$ years in the case of mepacrine, quinine, sulphadiazine and 3349† respectively, without any change developing in the parasites' susceptibility to the compounds used (Williamson and Lourie, 1947).

As we have already mentioned (Williamson and Lourie, 1947), the potential significance of these findings, in regard to the use of paludrine in human malaria, is of obvious and of very considerable importance. We have been careful to stress, however, that much further work is necessary before it could be suggested that there may be a serious danger of the development and propagation of paludrine-resistant strains of human malarial parasites as a result of the wide-spread use of paludrine in the field. Apart from the necessity of establishing whether human malarial species share with *P. gallinaceum* the possibility of acquiring paludrine-resistance, it is important to find out whether the development of such resistance would be favoured by the conditions under which paludrine will be (and is being) used in the field. Clearly, paludrine would be prescribed under two distinct circumstances, (a) when parasites are easily demonstrable in the blood, in which case

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† May and Baker Research Fellow.

‡ 2-*p*-Chlorophenylguanidino-4- β -diethylaminoethylamino-6-methylpyrimidine dihydrochloride (Curd, Davey and Rose, 1945).

the immediate purpose of treatment would usually be to alleviate the symptoms of infection, and (b) when parasites are not easily demonstrable in the blood, in which case the drug would be given either as a suppressive (implying that the infection is held in check without being eradicated), or as a causal prophylactic or radical curative agent (implying that the parasites are prevented from obtaining any initial foothold, or are completely eliminated after having established an infection). Under circumstances *a* the operative impact of treatment falls on the asexual blood forms of infection. It was by the treatment of these forms of *P. gallinaceum* that resistance was produced in our earlier work. Under circumstances *b*, where causal prophylaxis or radical cure is the object, treatment is aimed at the exo-erythrocytic forms, whose occurrence in human malaria is now no longer open to question (Shortt, Garnham, Covell and Shute, 1948; Shortt and Garnham, 1948). The present contribution records an attempt to produce paludrine-resistance by treatment directed essentially against the exo-erythrocytic forms of *P. gallinaceum* infection in chickens.

EXPERIMENTAL METHODS AND RESULTS

Davey (1946*b*) has shown that, although paludrine is capable of destroying the pre-erythrocytic stages of *P. gallinaceum*, it has only a limited action against the later exo-erythrocytic stages. Thus, he found that oral doses of 3 mgm. per 50 gm. body-weight administered twice daily to young chicks for five days will cure all sporozoite-inoculated birds, provided that treatment starts not later than 48 hours after inoculation; many cures can still be obtained if treatment does not start till 80 hours, but none if it starts more than 96 hours after inoculation. Lower doses are even less effective. Thus 0.25 mgm. per 50 gm. body-weight administered twice daily for six days fails to destroy even the pre-erythrocytic stages, and merely delays death for a few days; dosages up to 1.5 mgm. per 50 gm. body-weight twice daily fail to cure (i.e., fail to eradicate the later exo-erythrocytic stages) even if continued for as long as a month.

This limitation of paludrine's curative properties affords the opportunity of subjecting the exo-erythrocytic stages of the parasite to long-continued treatment in an infected chicken. By subinoculating large amounts of blood at any desired time during this treatment it is generally possible to obtain, and then to study in appropriate manner, the progeny of the treated exo-erythrocytic parasites.

Three chickens, 3-4 months old, nos. 4933 (Rhode Island Red), 4944 (Brown Leghorn), and 4975 (Rhode Island Red), weighing 650, 600 and 810 gm., were infected by mosquito-bite on May 21st, May 27th and May 23rd, 1947, respectively. The usual acute parasitaemia was observed, and parasites were last seen in blood films 17, 12 and 16 days respectively after the date of infection. On June 11th, 1947—that is, 20, 15 and 19 days since infection, or three days in each case since parasites were last seen—paludrine treatment by stomach-tube was begun, at a dosage of 0.25 mgm. per 50 gm. body-weight, administered twice daily six days per week and sometimes once on the seventh day. This was continued uninterruptedly for 25 weeks (when death from an unknown cause supervened) in bird 4933, for 41 weeks in bird 4944, and for a full year in bird 4975. The two latter birds were in excellent condition and weighed 1,200 and 2,100 gm. respectively at the end of their treatment-courses. At intervals throughout treatment 10-12 c.cm. blood was withdrawn from each of the three treated birds and subinoculated intravenously into fresh chickens.

TABLE I

Results of periodical subinoculation of 10-12 c.cm. blood from three mosquito-infected chickens during courses of paludrine treatment lasting up to one year

Since start of treatment		Bird no. 4933	Bird no. 4944	Bird no. 4975
Days (weeks)	No. of doses (0.25 mgm. per 50 gm. body-weight)			
41 (6)	69	+	+ <i>t</i>	+
66 (9)	112	—	—	+ <i>t</i>
91 (13)	156	+	—	+ <i>t</i>
122 (17)	214	+ <i>t</i>	—	—
150 (21)	266	+ <i>t</i>	—	—
178 (25)	318	Dead	—	+ <i>t</i>
206 (29)	368		—	—
237 (34)	422		—	—
285 (41)	500		—	+
347 (50)	600			+ <i>t</i>
365 (52)	634			+ <i>t</i>

+ = Subinoculation resulted in infection.

— = Subinoculation did not result in infection.

t = Parasites of subinfections tested for paludrine-resistance.

Table I shows the results of these subinoculations, and it seems that bird 4944 was cured at some time between the sixth and ninth week of treatment. Birds 4933 and 4975, on the other hand, retained their infections throughout treatment. At the stages indicated in the table by the symbol *t*, tests were performed in order to determine whether any resistance had developed. These tests were carried out by transferring the infections intravenously to well-grown birds, in order to obtain a suitable number of parasites at the stage of acute parasitaemia for the subsequent inoculation of six-day-old chicks, in which drug activity was assessed according to the method described by Davey (1946a).^{*} All the tests showed that the parasites derived from the long-treated, latently infected birds had failed to acquire any resistance to paludrine. It is unnecessary to present the actual records of each of these tests, since they were all carried out in exactly the same way, and all gave the same result. Only one example need therefore be given, and Table II

TABLE II

Response to paludrine of (a) parasites derived from a latent infection treated with paludrine for one year, and (b) parasites of the normal parent strain of *P. gallinaceum*

Parasites	Results†		
	0.5 mgm.	0.25 mgm.	0.1 mgm.
(a) From treated latent infection* ...	0/371	84/371	281/371
(b) From the parent strain ...	0/378	86/378	265/378

* In bird 4975, after 634 doses in 365 days (see Table I).

† The results are given as in the contribution by Davey (1946a). Treatments were by stomach-tube at above dosages per 50 gm. body-weight, twice daily for 3½ days, starting on the day of inoculation. The numerator is the average count of parasitized cells per 500 R.B.C.s in six treated chicks on the fifth day of infection, and the denominator is the average count on the same day in six untreated control chicks.

* In preparing appropriate inocula for the six-day-old chicks, we found it convenient and time-saving to use a simple nomogram, described elsewhere in this issue (Williamson, 1948).

shows the findings in the test on parasites obtained from bird 4975 after its infection had been subjected to 634 doses of 0.25 mgm. per 50 gm. body-weight during a period of 365 days.

The failure of paludrine-resistance to emerge as a consequence of the treatment of latent infections for periods up to a year is borne out not only by the tests on parasites derived from the three main infections of the experiment, shown in Table I, but also by further exactly similar tests carried out on parasites derived from two infections not mentioned above and not represented in the table. After 150 days of treatment (266 doses) in bird 4933, another fowl, no. 5027, was inoculated from that bird. Similarly, after 178 days of treatment (318 doses) in bird 4975, no. 5096 was inoculated. The two resulting infections, in nos. 5027 and 5096, were then allowed to pass through the stage of acute parasitaemia, and, when parasites were no longer to be found on microscopical examination, they were treated twice daily in exactly the same manner as the three main birds of the experiment. On the 217th day of infection (after 359 doses) in bird 5027,

TABLE III

Attempts to produce paludrine-resistance by treatment (a) during a latent infection, and (b) during the early stages of serially subinoculated infections*

Method	No. of infections treated	Intensiveness of treatment		Duration of treatment	Aggregate treatment		Result
		Average no. of doses per week (0.25 mgm./50 gm. body-weight per dose)	Average paludrine per week (per 50 gm. body-weight)		No. of doses (0.25 mgm./50 gm. body-weight per dose)	Total paludrine (per 50 gm. body-weight)	
a	1 (in bird 4975)	12.2	3.0 mgm.	52 weeks	634	158.5 mgm.	No acquired resistance
b	8 (seriatim)	4.9	1.2 mgm.	11 weeks	54	13.5 mgm.	Considerable acquired resistance

* See Williamson and Lourie (1947), Table III, p. 283.

and on the 85th day (after 118 doses) in bird 5096, the parasites were transferred to other birds by the usual intravenous inoculation of 10–12 c.cm. blood, and then tested as above for drug-resistance. The parasites of bird 5027 had therefore, at the time of their second resistance-test, been subjected during latency to a total of 625 doses over a period of 365 days in the successive infections of birds 4933 and 5027; whilst the parasites of bird 5096 had, in similar manner, at the time of their resistance-test, been subjected during latency to a total of 436 doses over a period of 263 days in the successive infections of birds 4975 and 5096.

Table III contrasts the failure to produce resistance by treating latent infections, as described above, with the ready acquirement of resistance by treating overt blood infections, as described in our earlier contribution (Williamson and Lourie, 1947). The table shows that the latent infection of bird 4975 was treated for about five times as long, and with about 12 times as much paludrine, as the blood infections which yielded highly resistant parasites in our earlier work.

DISCUSSION

GENERAL BIOLOGICAL CONSIDERATIONS

The failure to produce paludrine-resistance by prolonged treatment of latent infections, compared with the facility with which it can be produced by treating acute infections, is open to two main interpretations, *a* and *b* below.

(*a*) It is a general rule that the exo-erythrocytic stages of malaria parasites are much more resistant to drug treatment than are the erythrocytic asexual stages. Indeed, this is the root of the whole problem of successful antimalarial chemotherapy. In spite of paludrine's high degree of causal prophylactic activity (Curd, Davey and Rose, 1945; Davey, 1946*b*), this compound shares with other antimalarial agents their general inability to eradicate the later exo-erythrocytic forms of malarial species. Since these exo-erythrocytic forms, unlike the asexual blood stages, are relatively indifferent to the influence of paludrine, this should then provide an adequate explanation for the fact that plying these forms with paludrine fails to impose any resistance upon their erythrocytic progeny. It seems reasonable to suppose that drug-resistance, as an effect produced by drug treatment, can only arise, or can only arise readily, where the treatment is administered to a developmental stage of the parasite which is significantly susceptible to treatment.* The exhibition of paludrine to the asexual blood stages readily results in resistance, and here the drug has been presented to the parasites when they are highly susceptible to treatment. In a latent infection, however, the drug is presented to parasites which are relatively indifferent to, and uninfluenced by, the treatment, and it would therefore be unreasonable to expect this to result in the imposition of any acquired drug-resistance, or of any other change, on the descendants of the parasites treated.

(*b*) The other possible interpretation of the failure to produce resistance by treating latent infections derives from a likely theory as to the mechanism by which drug-resistance does appear in a strain of treated parasites. This theory is that resistant individuals occur naturally and spontaneously as rare mutants in a normally sensitive parasite strain, in the absence of any treatment whatever. Ordinarily these exceptional individuals fail to assert their presence because of the overwhelming preponderance of the normal sensitive type. The effect of treatment, however, is to provide an environment in which the latter are destroyed, and the rare resistant mutants selectively permitted to survive. These mutants, by definition, have the property of transmitting their resistant character to their descendants, and a stable drug-resistant strain is thus established.

There need be no difficulty about accepting the thesis that mutations constantly occur among parasitic protozoa, since this has been well established for even lower forms of life, i.e., bacteria. Dubos (1945) quotes the work of Lewis (1934) as an outstanding demonstration in this connection, and he points out that the first examples of adaptation to the utilization of a new substrate by bacteria were described by their discoverers as cases of mutation (Neisser, 1906; Massini, 1907). Luria and Delbrück (1943) have shown convincingly that the development of bacteriophage-resistant *Escherichia coli* is attributable to the selection of resistant bacteria which have spontaneously arisen by mutations of sensitive cells independently of the virus, along the lines of the theory out-

* This does not mean that the only way in which drug-resistance can be produced experimentally is by exposing the organisms to drug treatment. McIlwain (1943) has, for example, produced pantooylaurine-resistant bacteria by the simple process of training the originally sensitive organisms to grow in diminishing concentrations of the essential metabolite, pantothenic acid.

lined above for the development of drug-resistant parasites. Demerec (1945) and Oakberg and Luria (1947) have made a strong case for the explanation of acquired penicillin-resistance and sulphonamide-resistance respectively in *Staphylococcus* along these lines. It is, then, at least a highly plausible hypothesis that paludrine-resistance in *Plasmodium* arises by the same mechanism. In that case, resistance would be expected to appear much more readily when infections are treated in the stage of acute parasitaemia than in the stage of latency, for the simple reason that under the former circumstances there is a vastly greater parasite population subjected to the influence of the drug, with consequently greater chances for the selection and ascendancy of resistant mutants.

RELATIONSHIP TO HUMAN MALARIA

We have already stressed the necessity for caution in applying to human malaria the lessons of these investigations on *P. gallinaceum*. If, however, the relative ease with which paludrine-resistant strains can be produced in *P. gallinaceum* points to the danger that the wide-spread use of paludrine may readily give rise to resistant strains of human malaria parasites, then the present contribution may be regarded as a mitigation of the extent of that danger. A very high proportion of the paludrine used for treatment in man will be administered daily, or every few days, for long periods of time as a prophylactic or suppressive, either to forestall infection or to deal with the parasite remainder after the subsidence of overt infection. We know already that in the case of *P. vivax*, if not in *P. falciparum*, paludrine cannot be depended upon invariably to prevent or to eliminate infection (Maegraith *et al.*, 1946; Fairley *et al.*, 1946). There will therefore be very considerable numbers of infected individuals at large in whom the parasites (in the exo-erythrocytic form) are relentlessly subjected, day after day or week after week, to the influence of paludrine. These would be ideal circumstances for the production of paludrine-resistance, if it were the case that treatment applied to latent infections (that is, essentially, to exo-erythrocytic parasites) could readily give rise to such resistance. The present work shows, however, that this is not the case in *P. gallinaceum*, and it is probably permissible to expect that it may also not be the case in human malaria.

SUMMARY

1. The first paper in this series described the production of paludrine-resistant *P. gallinaceum*, within $2\frac{1}{2}$ - $3\frac{1}{2}$ months, as a result of treatment in the earliest stages of syringe-passaged infections, when the full impact of treatment must fall on relatively large numbers of erythrocytic asexual forms of the parasite. The present paper reports failure to produce resistance when the treatment is administered to latent infections (i.e., after the phase of acute parasitaemia), even if the treatment, twice daily, be continued for as long as a year; treatment under these circumstances necessarily falls mainly, if not exclusively, upon the later exo-erythrocytic stages of development. It is remarkable, incidentally, that a bird can retain its infection for a whole year in spite of persistent daily dosage with paludrine.

2. Failure to produce resistance by treating latent infections is susceptible of at least two explanations:

(a) Since the later exo-erythrocytic forms are relatively uninfluenced by paludrine, it would perhaps be unreasonable to expect any acquired resistance, or any other change, to be imposed on the descendants of parasites treated during that phase of the life-cycle.

(b) A likely mechanism for the development of drug-resistance is that resistant individuals occur spontaneously as rare mutants in a normally sensitive parasite strain, in the absence of any treatment whatever. Ordinarily they are unable to assert their presence because of the overwhelming preponderance of normal individuals. Drug treatment, however, provides the conditions for selective survival of these mutants, and a stable drug-resistant strain thus emerges. The chances of this selective process coming into effect are considerably higher where treatment is directed against the vastly greater numbers of parasites in an overt blood infection than where it is applied to the relatively small numbers present in a latent infection.

3. A very high proportion of the paludrine used for treatment in man will be administered daily, or every few days, for long periods of time as a prophylactic or suppressive. Under these conditions there will be very considerable numbers of individuals taking paludrine regularly while harbouring latent infections, at least of *P. vivax* if not of other forms of malaria. The circumstances would be ideal for the development of paludrine-resistance, if resistance could, in fact, easily be produced by subjecting exo-erythrocytic parasites to the influence of the drug. The results described above suggest, however, that the danger of giving rise to paludrine-resistant strains of malaria in this way is insignificant.

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OBSERVATIONS ON THE OCCURRENCE OF LARVAE OF *OESTRUS OVIS* IN THE NASAL CAVITIES AND FRONTAL SINUSES OF GOATS IN NIGERIA

BY

K. UNSWORTH

(*Veterinary Education Officer, Vom, Nigeria*)

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The occurrence of larvae of *Oestrus ovis* in the nasal cavities and sinuses of goats in West Africa has already been recorded (Roubaud, 1914). Reference is also made to the condition in the annual report of the Veterinary Department, Nigeria, for 1937, where the statement is made that 'a nasal myiasis due to the larvae of the bot-fly *Oestrus ovis* is common in goats and sheep in the late rainy season.' This undoubtedly refers to the occurrence of more mature larvae, since the report goes on to describe the symptoms associated with their presence. Perusal of the available literature subsequent to this date has failed to reveal any further mention of the condition in Nigeria, and no figures appear to be available regarding the percentage infestation-rate among goats at any period of the year. It is considered appropriate, therefore, in view of possible future developments in the live-stock economy of the Colony, to draw attention once more to the existence of the parasites, particularly in view of the high infestation-rate among goats brought in from certain areas.

The goats which have, so far, been examined—220 in all—were brought from Kano and from Kafo, which is situated on the plateau a few miles from Vom. The nasal chambers and the frontal sinuses were searched for the presence of larvae, after the bony structures of the skull had been sawn through longitudinally in the mid-line. The maxillary sinuses were not examined, so that the infestation-rate recorded below probably does not represent the maximum. The examinations were made during the month of May, 1948.

Among 100 goats brought from Kafo, 19 (19 per cent.) were found to be infected; of 120 from the Kano area, 78 (65 per cent.) were found to have one or more larvae in the sites examined. The majority of larvae were very young forms, although a small number of mature larvae were encountered. This would appear to suggest that the period during or just prior to that at which the examinations were made was associated with considerable adult-fly activity, particularly in the Kano area, where the climate at that time of year is hot and dry—a concept which would fit in with the presence, referred to above, of mature larvae in the nasal passages of goats towards the end of the rainy season.

Of the mature larvae encountered during the course of this survey, four were placed on the surface of dry soil in a glass jar. They immediately penetrated below the surface and pupated, and adult flies have subsequently emerged, the length of the pupal period being 25–26 days. Examination of the soil contents of the jar on the 28th day revealed four empty pupa cases and one dead malformed adult fly, which had apparently been unable to reach the soil surface.

The precise economic importance of this parasite in Nigeria is difficult to assess at this preliminary stage of the investigation. Although there is a large goat population, the

animals occur in scattered, generally small, herds, and there is no attempt at goat-farming in the accepted sense of the term. The parasite may, however, become a serious pest should goat-farming, and, more especially, sheep-farming, be adopted on an extensive scale, and it is with the object of focusing attention on a potential hazard that this article is presented at this early stage.

ACKNOWLEDGEMENTS.—This article is published with the kind permission of the Director of Veterinary Services, Nigeria.

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MISCELLANEA

THE BREEDING-PLACES OF *CHRYSOPS SILACEA*

The habitat of the pre-adult stages of *Chrysops silacea*, the principal vector of *Loa loa* in West Africa, has not previously been recorded. During 1947 one of us (I.J.C.) found two pupae of this species at Kumba in the British Cameroons. During June and July, 1948, we re-examined this area and demonstrated that the *Chrysops* breeding-places were confined to densely shaded areas of streams, where the larvae were found localized in mud, rarely more than three inches from the surface, lying under a layer of decaying leaves. A full account of the investigation, together with suggestions for possible means of control, will be the subject of a later communication, but this preliminary note is being published now in order to assist other workers in their search for the breeding-places of this important vector.

L. J. CHWATT,
R. M. GORDON,
C. M. JONES.

July 29th, 1948.

17 MAR 1949

ANOPHELINE LARVAE COLLECTED IN ARABIA

BY

H. S. LEESON

(From the London School of Hygiene and Tropical Medicine)

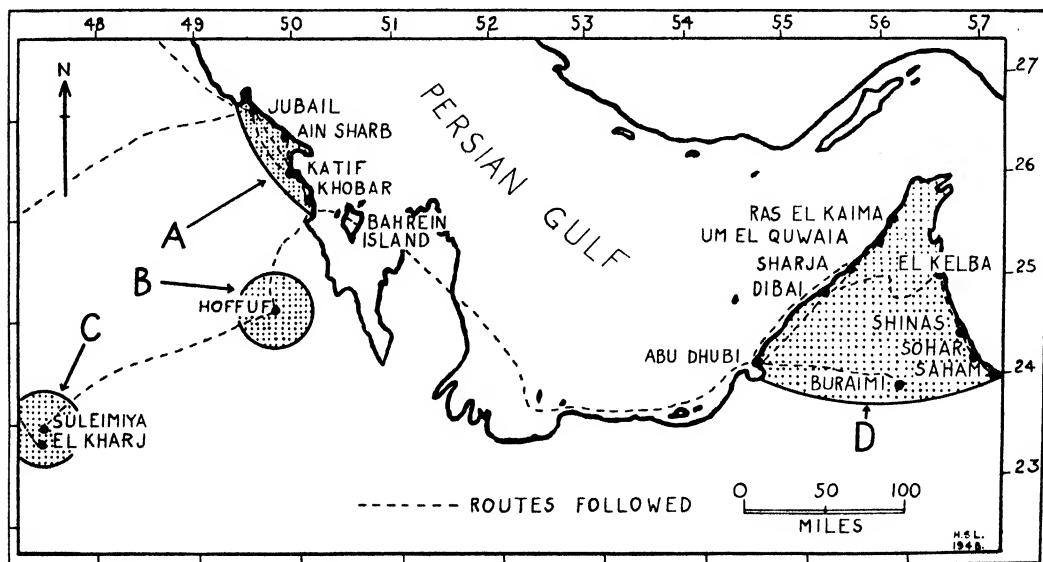
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During a malaria survey in Arabia in the winter of 1943-44 Major C. M. Hopkins, R.A.M.C., of No. 3 Malaria Field Laboratory, made a collection of adults and larvae of anopheline mosquitoes. The area surveyed was that part of Arabia north of the Tropic of Cancer along or near the coast of the Persian Gulf. Unfortunately the adults cannot now be traced, but the larvae were left for safe keeping in the London School of Hygiene and Tropical Medicine. The collection has been examined, and the opportunity is now taken to place the identifications on record.

The following 10 species have been identified: **Anopheles coustani* Lav., *A. hyrcanus* Pall., *A. fluviatilis* James, **A. pulcherrimus* Theo., **A. culicifacies* Giles, *A. adenensis* Christ., **A. sergenti* Theo., **A. multicolor* Camb., **A. stephensi* List., **A. cinereus* Theo.

The seven species marked with an asterisk are included in the list of anophelines recorded from Arabia given by Buxton (1944), who notes that Christophers and Puri (1931) examined a larval skin of the anopheline named *A. arabica* 'and concluded that it is not *funestus*, but probably near *fluviatilis*.' In the same paper Buxton also suggests that *A. hyrcanus* 'might occur in the north' and that 'it may be thought that *adenensis* is a distinct species.' Larvae of *A. fluviatilis* are fairly numerous in Hopkins's collection; a few *A. hyrcanus* larvae were found at Hoffuf, and Christophers's variety *adenensis* of *A. culicifacies* has recently been raised to specific rank by De Meillon (1947).

The places visited by Major Hopkins may conveniently be grouped as shown on the accompanying sketch-map (areas A, B, C and D).



SKETCH-MAP of part of Arabia, showing where Major Hopkins made his collections of anophelines, 1943-44.

In area A, around Katif, Khobar, etc., *A. stephensi* was the most numerous species taken in January, 1944. Larvae were found in almost any kind of water, the most important breeding-places being the drains in gardens and date-palm groves, the blind ends of which are never dry. Other breeding-places are wells and borrow-pits. *A. fluviatilis* larvae were collected from shaded fresh running water in drainage or irrigation canals, but at Saihat some larvae were found in brackish wells. Other larvae taken in this area, in order of frequency, were *A. sergenti*, *A. pulcherrimus*, *A. multicolor* and a few *A. coustani*.

Area B is around Hoffuf. *A. stephensi* and *A. fluviatilis* were common, and the same species were encountered here as in area A. In addition, there were a few larvae of *A. hyrcanus*. One larva taken at Hoffuf, among 70 larvae of *A. stephensi* and five of *A. multicolor*, has all the main characters of *A. cinereus*, the only apparent difference being that on the ventral side of the prothorax of this larva hair 13 (Puri, 1931) has three branches only, whereas in *A. cinereus* it usually has six or seven branches. No larvae of *A. cinereus* were present in collections made at other places.

Further inland, at Suleimiya and El Kharj in area C, *A. multicolor* was the only anopheline found in February. Larvae were taken from drains, irrigation canals and wells. Major Hopkins made special searches for other species but failed to find any, and at 11 other places in this area no anophelines were seen at all.

In most parts of the Oman and Trucial Oman (area D) *A. culicifacies* was the only species during March and April. The chief breeding-places were fresh-water wells. Larvae of this anopheline are not present in the collections made in the other areas. At El Kelba a few larvae were taken which possessed the longitudinal dark head-markings of *A. adenensis*. There were also some larvae of *A. stephensi* from El Kelba and Shinas.†

The following key is given for the identification of fourth stage anopheline larvae which might be expected to occur in this little-known region. Species seen in Major Hopkins's collection are marked with an asterisk.

- | | |
|---|-----------------------|
| 1. Inner anterior clypeal hairs with bases nearly touching | 2 |
| Inner anterior clypeal hairs with bases well separated | 3 |
| 2. Inner shoulder hairs branched into 3 or 4 near base | <i>coustani</i> * |
| Inner shoulder hairs branched into 3 or 4 near tip | <i>hyrcanus</i> * |
| 3. Main tergal plates exceptionally large, with hind margins convex | <i>fluviatilis</i> * |
| Main tergal plates not exceptionally large, with hind margins straight or concave | 4 |
| 4. Outer anterior clypeal hairs conspicuously branched with 4-12 branches | <i>pulcherrimus</i> * |
| Outer anterior clypeal hairs simple or merely frayed | 5 |
| 5. Two long mesopleural hairs, both branched | 6 |
| Two long mesopleural hairs, not both branched | 8 |
| 6. Abdominal palmate hairs on segments II-VII | 7 |
| Abdominal palmate hairs on segments IV-VII | <i>turkhudi</i> |
| 7. Antennal shaft-hair simple, fairly stout, not longer than width of shaft; hair 13 on ventral side of prothorax with 7-10 branches | <i>hispaniola</i> |
| Antennal shaft-hair simple, slender, and longer than width of shaft; hair 13 on ventral side of prothorax with only 3 branches† | <i>cinereus</i> * |

† Since these notes were written, the author has received some adult anophelines collected by Mr. R. H. Daggy, of the Arabian-American Oil Co., Dhahran, Saudi Arabia, between January, 1947, and February, 1948. The species taken in area A were *A. stephensi* from Khobar, Damman and Saihat, *A. fluviatilis* from Saihat, and *A. pulcherrimus* and *A. coustani* from El Ajam. From area C were *A. multicolor* and *A. stephensi*, both taken at El Kharj.

‡ There was only one larva of *A. cinereus* in Hopkins's collection. Other larvae of this species from Southern Rhodesia had six or seven branches on hair 13.

8. Two long mesopleural hairs, both simple 9
 Two long mesopleural hairs, one simple and one branched 11
9. Caudal hairs : ends of outer hairs curved, ends of inner hairs not curved 10
 Caudal hairs : ends of both inner and outer hairs curved *dthali*
10. Head-markings : 3 dark spots behind bases of frontal hairs *culicifacies**
 Head-markings : dark Y-shaped pattern, with stem behind bases of frontal
 hairs forking forwards to anterior border of clypeus *adenensis**
11. Head-markings : dark transverse band behind bases of frontal hairs ; meta-
 thoracic hair no. 1 developed as palmate hair *sergenti**
 Head-markings : dark spots, or head entirely dark ; metathoracic hair no. 1
 an ordinary hair or a poorly developed palmate hair 12
12. Head-markings : dark spots around bases of inner frontal hairs, or head
 entirely dark *multicolor**
 Head-markings : no dark spots around bases of inner frontal hairs 13
13. Metathoracic hair no. 1 fairly well developed as palmate hair ; inner anterior
 clypeal hairs frayed *superpictus*
 Metathoracic hair no. 1 as ordinary hair ; inner anterior clypeal hairs simple *stephensi**

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EXPERIMENTAL STUDIES ON THE THERAPY OF SCHISTOSOMIASIS

BY

WALTER KIKUTH

AND

RUDOLF GÖNNERT

(From the Chemotherapeutic Institute of the Farbenfabriken Bayer, Elberfeld, Germany)

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INTRODUCTION

The first successful treatment of schistosomiasis was by means of tartar emetic (Christopherson, 1918). This preparation, however, has the disadvantage of causing frequent side-effects and marked local irritation if even a small amount of emetic be injected outside the vein. Consequently, a more satisfactory antimony preparation was sought for, and was found by Dr. H. Schmidt in fouadin, which is much less toxic than tartar emetic and can be given intramuscularly. Results obtained with antimony preparations have been exhaustively reviewed by Schmidt and Peter (1938). Emetine has also been recommended, but it is less active than the antimony preparations and requires intravenous administration. Thus, in spite of good results obtained with fouadin and tartar emetic, treatment still remains unsatisfactory, and in mass treatment frequent parenteral injections are necessary, which require a large staff of doctors and auxiliary personnel. The need for an effective remedy which can be given by mouth led us to establish a model technique for chemotherapeutic investigation by means of which chemical preparations can be tested on a wide basis.

When the work was begun in 1932 there had been little chemotherapeutic investigation on animals infected with schistosomiasis, and such work as had been done was concerned chiefly with *Schistosoma japonicum* infections in larger animals. For our purpose, however, *S. japonicum* infections were not practicable, since at that time it was not possible to maintain and breed the snail vectors in Germany. Work with *S. mansoni*, however, seemed to offer a promising line, for the parasite develops regularly to the adult stages in white mice, which are particularly suitable for large series of investigations, and excretion of the eggs in the faeces enables a regular control to be kept of the infection in mice, as in man. Moreover, it had been shown at the Institut für Schiffs- und Tropenkrankheiten at Hamburg that the complicated life-history of schistosomes could be completed under experimental conditions in the climate of Germany. We therefore chose *mansoni* infections of white mice as the basis for our chemotherapeutic studies, and by 1936 the preliminary work was so far complete that therapeutic trials could be commenced.

MATERIALS AND METHODS

The *S. mansoni* strain used was kindly provided by Professor Vogel, of the Institut für Schiffs- und Tropenkrankheiten, Hamburg. It was first maintained in two chronically infected rhesus monkeys, in the stools of which *S. mansoni* eggs could regularly be demonstrated. The vector snail was *Planorbis guadeloupensis*, which was also kindly

provided by Professor Vogel in 1936 and has lived and multiplied well in our aquaria. Egyptian *Planorbis* and *Bulinus*, which Professor Khalil of Cairo kindly sent to us, could not be infected by *mansoni* strains from Liberia.

The snails, *P. guadeloupensis*, were maintained in medium-sized aquaria, about 30 by 22 by 22 cm. or 60 by 26 by 28 cm. in size, which were provided with a layer of sand and gravel and planted with water-plants. A water-temperature of 26–28° C. was satisfactory. It is important to give the snails an ample food-supply of lettuce, supplemented by animal food, shrimps, liver and earth-worms. The water should be filtered through a charcoal filter to avoid putrefaction. Although intensive irradiation with ultra-violet light was necessary at the beginning of the cultivation of the snails, they later became so acclimatized that illumination was less important.

In order to infect the snails, faeces containing eggs of *S. mansoni* are collected and washed several times in 2-3 per cent. saline, the sediment being allowed to settle each time. The eggs settle quickly, so that the saline can be renewed after 10-20 minutes. When the washings become clear the sediment is washed once again in iced water. The washed eggs are then kept overnight in the ice-chest. On the next day the iced water is replaced by water at about 40° C., and the glass vessel containing the eggs is placed under strong electric illumination. The miracidia quickly emerge under the influence of the warmth and light, and, after a little practice, can easily be seen with a pocket-lens as white threads swimming quickly about. This technique for obtaining miracidia to infect snails is similar to that used in the clinic for the examination of faeces in schistosome infections—the so-called *Schlüpfversuch*. The snails to be infected are placed in the water containing the miracidia, one snail being allowed for every 30 schistosome eggs. To facilitate this a count is made of the number of eggs in a given quantity of faeces-sediment, after the iced water has been poured off and before the warm water has been added.

When only a few animals are used, the infected snails are maintained in the same way as uninfected snails, preferably in aquaria with filtered water or in large flat dishes. At 26–28° C. the development of the schistosomes in the snails as far as cercariae lasts from four to seven weeks. If the snails are heavily infected, or if their living conditions are unsatisfactory, many die from the infection. On the other hand, less heavily infected snails carefully maintained can live up to three months and daily liberate hundreds or even thousands of cercariae. The cercariae come out only about midday, and then only after intensive illumination and warming of the water. Accordingly, when cercariae are required, the snails are put into small vessels which are placed under a strong source of warmth and light (a Sollux lamp). To count the cercariae the water is stirred well, three samples of 1 c.cm. are mixed with formalin, and the dead cercariae are counted. When definite numbers of cercariae are required—e.g., for the infection of a vertebrate host—the average of these three counts is taken. This is sufficiently accurate for our purposes. Since dipping the experimental animal into water containing cercariae leads to very irregular infections, we inject the cercariae subcutaneously. This seldom leads to inflammation or abscesses, in spite of the large number of bacteria contained in the water. The cercariae from as many snails as possible are mixed together in order to avoid unisexual infections. Mice are usually infected with 50–60 cercariae, monkeys with 200–300. For routine investigations we use mice, and for special studies monkeys especially rhesus monkeys.

The young schistosomes reach sexual maturity in about six weeks and eggs appear in the faeces by the 48th day. At this stage the mice used for chemotherapeutic testing are given one dose of the substance to be tested daily for six successive days, except on Sundays, when the interval is 48 hours. We seldom tested the compound by parenteral injection, since the aim of our investigations was to discover a drug effective by mouth. A control examination of the faeces before treatment is not necessary, since, although only a small number of cercariae develop into mature worms, failures, or infections only with males or females, are exceptional. By our technique, the number of mature schistosomes varies from two to 12 pairs. If the substances have no effect, the mice are used again, for the testing of another preparation, after not less than four weeks from the beginning of the experiment. In order to investigate whether effective preparations also kill young worms (i.e., those sexually immature), mice are treated from the 33rd day after the infection. With monkeys treatment is not begun until eggs appear in the faeces. Since it was impossible to obtain a sufficient number of monkeys during the war, we were compelled to use the same ones several times over. This caused no difficulty if they had first been treated with a non-effective substance or with ineffective doses, since the excretion of eggs continued without interruption and the worms were not damaged. If, on the other hand, the first treatment had been successful, the monkeys were kept under observation for at least three weeks, and, if there was no relapse during that time, they were re-infected, and examined frequently during the following six weeks, i.e., when the new group of schistosomes had become sexually mature. If the faeces remained negative during that time—i.e., for at least nine weeks in all—the monkeys were used for a new investigation.

The excretion of eggs in the faeces served as an indication of infection. For our purposes a quantitative measurement of the excretion of eggs was not necessary, and we were satisfied merely with determining their presence or absence. It was sufficient to examine the faeces only twice weekly, the *Schlüpfversuch* technique, described above, being used.

CRITERIA FOR THE DETERMINATION OF THERAPEUTIC ACTIVITY

In order to prove the activity of a substance we used various criteria. In the case of the living animal we depended exclusively on the excretion of eggs. The count of eosinophils in the blood was of disappointingly little value, because the mice were frequently infected with other helminths. From our experience with active antimony compounds and later with the miracid group, we consider a compound to be inactive if excretion of eggs continues for three weeks after the end of treatment and shows no irregularities during that time. Occasionally, one examination of the faeces may accidentally fail to show eggs during a long series of positive examinations. The activity of a substance can therefore only be demonstrated in the first place by the consistent absence of eggs from the faeces.

Further evidence can be obtained by autopsy, which was undertaken if no relapse occurred for 4–10 weeks after the disappearance of eggs. Special attention was paid to the following points: the presence and site of living worms; the condition of dead worms; the number and condition of eggs in the liver; and the amount and distribution of schistosome pigment in the liver. While mature healthy worms reside in the loops of the intestinal veins, damaged and immature worms are found in the branches of the portal vein in the liver. Dead worms are carried by the blood-stream into the liver veins,

often as far as the peripheral parts of the hepatic lobes, where they are apparently broken down by digestive enzymes of the organism or by autolytic processes. They are then best found by crush-smears of the liver. Their resorption proceeds only very slowly, and as a rule 8-10 weeks after treatment easily recognizable remains of them are still to be found in a connective-tissue capsule, which may be surrounded by heavy deposits of pigment. According to the degree of disintegration of the worms and the interval since the end of treatment, conclusions can be drawn of the speed with which the substance exerts its activity. In the same way, the eggs which have been swept along into the liver, and which vary in number according to the severity of the infection, produce a characteristic picture. Macroscopically they appear as small white foci, similar to tubercles and consisting of connective tissue surrounding the eggs, which block the capillaries. If there is much infarction by the eggs, as in severe or chronic infections, much granulation-tissue is formed, which may lead to necrosis of parts of the parenchyma, giving a picture of cirrhosis. Different stages of developing cirrhosis are seen according to the age and severity of the infection. Since the life of the eggs lasts only a few weeks, all transitions are found, from eggs with living embryos down to empty remains of the egg-shells. After successful treatment there are only old foci of eggs and fusions of egg debris encapsulated by connective tissue. Consequently the finding of eggs in the liver is a valuable index of the activity of a preparation, since the discovery of a single living or well-preserved egg more than five weeks after the end of treatment is an indication of the presence of living worms.

Attention should also be paid to the brownish-black schistosome pigmentation of the liver. In early infections this occurs in all Kupffer cells, the distribution inside a cell being diffuse. The more advanced the infection, the greater is the tendency to concentration and clumping of the pigment in the individual cells, together with a strong deposition of pigment, mostly in the connective-tissue capsules around the eggs. The pigment is a metabolic product of the worms. Accordingly, when the parasites have died the new formation and the diffuse depositions of the pigment cease. At this stage, therefore, only clumped pigment can be seen, which is gradually broken down and slowly disappears. If the autopsy is carried out at least four weeks after the cessation of egg-excretion in the faeces, the presence of traces of a diffuse pigment is as much an indication of the existence of living worms as the finding of single living eggs. Both the microscopic picture of the liver and its macroscopic appearance depend on the quantity and distribution of the pigment. With intense infections the liver has a brown to brownish-black colour; with older infections it becomes more greyish-black, owing to the connective-tissue changes produced by the eggs. When the deposition of the pigment ceases, the liver gradually becomes clearer again and the cirrhotic changes become more prominent.

EVALUATION OF ACTIVITY

The activity of a substance was evaluated as follows.

1. *No action*, which was indicated by the excretion of eggs being continued for at least three weeks (*Schlüpfversuch* technique, or microscopical control).
2. *Slight action*, which was indicated by interruption or brief cessation of egg excretion, and at autopsy by mature worms being accompanied by dead ones or by worm-debris.
3. *Action*, which was indicated by cessation of egg excretion for at least three weeks

followed by relapse, and at autopsy by most of the worms being dead and disintegrating, though there might be rare living worms or living eggs, and diffusely distributed pigment might be found in crush preparations.

4. *Cure*, which was indicated by the faeces becoming free from eggs, with no relapse, during an observation-period of at least six weeks, and at autopsy by no trace being found of living worms or living eggs, and by no diffusely distributed pigment being found in crush preparations of the liver.

In considering the autopsy findings in mice, it is necessary to remember that a definite conclusion can be made only 3-4 weeks after the end of treatment, and that, in mice which die naturally, autolytic changes occur very rapidly within a few hours, which may give the appearance of degeneration due to chemotherapeutic activity.

DISCOVERY OF THE MIRACIL SERIES

By means of the technique described above, compounds active against human infections of *S. mansoni*, such as tartar emetic, fouadin and emetine, were found to be active also against *S. mansoni* infections in mice, as Oesterlin (1934) has already stated. A large series of other chemical compounds were concurrently examined for activity against *mansoni*, and in August, 1938, we found a new active compound, Ms. 577, which was subsequently named miracil A. Miracil A (1-diethylaminoethylamino-4-methylxan-

TABLE I
Toxicity of the miracil compounds after administration of one single dose by various routes

Route	Miracil A		Miracil B		Miracil C		Miracil D	
	Lethal	Non-lethal	Lethal	Non-lethal	Lethal	Non-lethal	Lethal	Non-lethal
By mouth ...	10.0	6.6	28.6	20.0	13.3	10.0	20.0	13.3
Subcutaneously ...	10.0	6.6	133.3	100.0	2.0	1.3	10.0	6.6
Intravenously ...	1.0	0.7	2.0	1.3	1.0	0.7	5.0	3.3

Doses are given as mgm. per 20 gm. mouse.

thone) was synthesized by Dr. Mauss, in the Wissenschaftlich-Chemisches Laboratorium of the Bayer Research Station at Elberfeld, and belongs to the xanthone series. Of the chemotherapeutic activity of this series nothing had previously been known.

The joint researches were continued, on the chemical side by Dr. Mauss and on the biological side by ourselves. Three other compounds of this series, named miracil B, C and D, were later found to be particularly noteworthy; their formulae are given below. The chemical aspects of this work on xanthone and thioxanthone derivatives will be described by Dr. Mauss in a separate communication.

The toxicities of one single dose of the compounds for mice are given in Table I. When given by mouth, all four miracils have approximately the same toxicity; after subcutaneous administration, however, there are considerable differences. The remarkably good tolerance of miracil B when given subcutaneously is, in our opinion, due to poor absorption, since the preparation is precipitated almost immediately after injection, and can easily be recognized weeks later under the skin by its particularly intense yellow colour. In contrast, miracil C is apparently well absorbed, as may be concluded from its high toxicity when given subcutaneously, which approximates to the intravenous toxicity of miracil

TABLE II
Results of treatment of mice by six successive doses of the miracil compounds

Route	Miracil A	Miracil B	Miracil C	Miracil D
By mouth	C, A 3.3 A 2.5 TrA 2.0 O 1.7	C 5.0 C 2.5 C 1.3 A 0.7 TrA 0.33 O 0.17	C 3.6 C 1.8 C 1.3 TrA 1.0 O 0.7	C 2.5 C, A 1.3 A, TrA 0.7 O 0.33
Index	1 : 1.7	1 : 15	1 : 3.5	1 : 4
Subcutaneously ...	C, A 3.3 TrA 2.5 O 1.7	C, A 2.5 A 1.3 TrA 0.7 O 0.33	A 1.3 TrA 0.7 O 0.33	C, A 2.5 A 1.3 O 0.7
Index	1 : 1.3	1 : 4	1 : 2	1 : 2
Intravenously ...	O 0.25	O 0.5		A, TrA 1.3 O 0.7
Index	1 : 0	1 : 0		1 : 1

Doses are given as mgm. per 20 gm. mouse.
C = Cure.
A = Action.
TrA = Trace of activity.
O = No action.

A, B and C. The toxicity of miracil D when given intravenously is remarkably low in comparison with its toxicity after oral and subcutaneous administration and in comparison with miracil A and B.

On repeated administration the individual tolerated dose is smaller than when treatment is by a single dose only. Tolerated doses for administration on six days are given in Table II, in which the highest amounts of the preparation given are the tolerated doses.

Detailed investigations of the miracils in mice has led to the following conclusions.

1. *The most favourable method of administration is by mouth* (see Table II). A comparison of the chemotherapeutic indices shows the superiority of oral administration. The good index of miracil B when given subcutaneously depends on the poor absorption of this preparation, already mentioned, which leads to a diminution of the toxicity and simultaneously to a depot-effect, with considerable local irritation.

2. *Even a single dose can effect a cure* (see Table III). Table III shows the total amounts, in mgm. per 20 gm., by which cure can be produced by the oral administration

TABLE III
The total amounts by which cure can be produced in mice by the oral administration of a single dose, or of six subdivided doses, of the miracil compounds

Frequency of treatment	Miracil A	Miracil B	Miracil C	Miracil D
6 times	20.0	30-7.5	21.4-10.7	15-7.5
Once	6.7	10-2.5	—	6.7-3.3

Doses are given as mgm. per 20 gm. mouse,

TABLE IV

Chemotherapeutic indices showing the relation between the effectiveness of the miracil compounds and the age of the worms at the beginning of treatment

Age of worms at beginning of treatment	Miracil A	Miracil B	Miracil C	Miracil D
Sexually mature (48 days after infection or later)	1 : 1.7	1 : 15	1 : 3.5	1 : 4
Immature (33 days after infection) ...	1 : 0	1 : 4	1 : 1	1 : 1

of a single dose or of six subdivided doses respectively ; it is evident that a smaller amount is required to produce a given effect if it is administered as a single dose than if it is subdivided into six daily doses.

3. *Miracil compounds are more active against sexually mature worms* than against immature ones (see Table IV). The figures in Table IV are chemotherapeutic indices ; they show the relation between the effectiveness of the miracil compounds and the age of the worms at the beginning of treatment.

The investigations in monkeys could not be carried out on the same broad basis as those in mice. To some extent this was unnecessary, for the experience obtained in the studies on mice could be used in the tests on monkeys. Accordingly, the preparations were given almost exclusively by mouth, but the lethal oral dose in monkeys could not be determined because large amounts of the compounds caused vomiting (see Table V). Table V summarizes the results of oral treatment. When a dose was repeated it was given at three-day intervals. The striking activity of miracil D is particularly noticeable. Miracil D also gave good results on subcutaneous administration ; cure was produced by two doses of 20 mgm. per kgm., and even by two doses of 10 mgm. per kgm. Two doses of 5 mgm. per kgm. were, however, ineffective.

The poor activity of the miracil compounds on immature worms shown in the mouse tests (Table IV) was confirmed in corresponding tests on monkeys (Table VI). These findings will be considered later, in the discussion on the mode of action of the miracil compounds.

TABLE V

Toxicity and activity of oral doses (in mgm. per kgm.) of the miracil compounds in schistosomiasis of monkeys

	Miracil A	Miracil B	Miracil C	Miracil D
Dose causing vomiting	200-100	100-75	300	800-400
Therapeutic doses ...	3 × 50 C 2 × 50 A 2 × 35 A 2 × 20 O	2 × 100 A 1 × 100 O	2 × 75 C 2 × 35 C 2 × 25 O	2 × 100 C 1 × 50 C 2 × 35 C 2 × 20 C 1 × 20 C 2 × 10 C 2 × 5 C, O 2 × 2.5 O

C = Cure.

A = Action.

O = No action.

PHARMACOLOGICAL INVESTIGATIONS OF MIRACIL D*

Investigations of miracil D, carried out in the pharmacological laboratory of the Bayer Research Station at Elberfeld by Dr. Hecht, yielded the following results.

1. *Local action.* 0.1 c.cm. of 1 per cent. solution injected intradermally into a rabbit's ear caused tissue necrosis; dropped into a rabbit's eye it caused oedematous-purulent conjunctivitis. 0.1 c.cm. of 2 per cent. solution injected intramuscularly into rats produced similar local oedema and necrosis.

2. *Toxic doses.* Orally, mice tolerated up to 0.3 gm. per kgm.; 0.5 gm. per kgm. killed half of the animals. Rabbits tolerated 0.2–0.6 gm. per kgm. without harm; after 0.7–1.0 gm. per kgm. there were no acute symptoms, but death ensued later. After intravenous administration of 20–25 mgm. per kgm., cats and rabbits showed convulsions, from which they recovered; 40 mgm. per kgm. was quickly fatal.

3. *Repeated administration.* In rabbits, repeated administration of daily doses of 0.05–0.1 gm. per kgm. by mouth, or of 20 mgm. per kgm. intravenously, caused lesions from which some of the animals died. These lesions included fatty degeneration of the liver, kidneys and heart. Cats were particularly sensitive, and could not tolerate daily doses of even 0.01 gm. per kgm. without ill effects; vomiting occurred frequently, and

TABLE VI
Effect of the miracil compounds on worms of different ages from monkeys

Age of worms at beginning of treatment	Miracil A	Miracil C	Miracil D
Mature	2 × 50 A	2 × 75 C	2 × 100 C
25 days after infection	2 × 50 O	2 × 75 O	2 × 100 A
15 days after infection	—	—	2 × 100 O

C = Cure. Doses in mgm. per kgm.
A = Action. O = No action.

even a week after treatment there was still a yellow discoloration of the aorta. Daily doses of 0.02 gm. per kgm. caused death after some delay. In cats there were the same fatty changes of the organs as in rabbits, but to a more marked extent.

Pharmacological investigations in monkeys could not be carried out as fully as we should have wished, but the following conclusions can be drawn from our chemotherapeutic experiments.

Single doses of 0.8 gm. and 0.4 gm. per kgm. caused vomiting, 0.4 gm. and 0.2 gm. per kgm. caused slight diarrhoea. Altogether 13 monkeys were treated with miracil D; three of these died, five, 12 and 16 months after treatment, almost certainly from causes other than intestinal irritation. During our period of observation slight loss of weight occurred in practically all the monkeys, but it is not clear whether this was a result of treatment, of repeated schistosome infections, or of insufficient nourishment owing to war conditions. Our observations suggest, however, that loss of weight can result from successful treatment—perhaps as a result of injury to the monkeys by toxins liberated from the worms and through embolism of the liver vessels by dead worms—a suggestion

* Articles on the pharmacology of miracil have also been published by English workers; see Wood (1947) and Hawking and Ross (1948).

which is supported by therapeutic experiments on old infections, in which loss of weight occurred only when the treatment produced a partial or complete cure. If the treatment was without effect (as is the case with immature worms) the weight of the monkeys remained unchanged. This was observed in the case of four monkeys treated with two doses of miracil D (100 mgm. per kgm.) and with two pairs of monkeys treated with miracil A and C respectively. With all three compounds the doses used were the highest which could be tolerated without definite gastro-intestinal irritation.

Our chemotherapeutic investigations in monkeys have shown no indication of definite toxic effects from miracil D. By analogy, it is possible that miracil D will be better tolerated by man than would be expected from the experiments on rabbits and cats reported above.

SUMMARY OF THE CHEMOTHERAPEUTIC RESULTS

Against experimental mouse schistosomiasis miracil B is clearly superior to the three other miracil compounds. It is almost four times as active as miracil C and D, and more than eight times as active as A. All the miracil compounds are well tolerated when administered by mouth. When given subcutaneously they produce marked local irritation, which occasionally leads to necrosis at the site of the injection. On intravenous injection they are inactive, or, in the case of miracil D, only slightly active. Pharmacological investigations also contra-indicate parenteral administration.

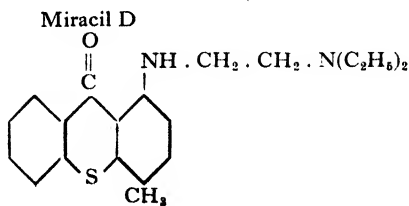
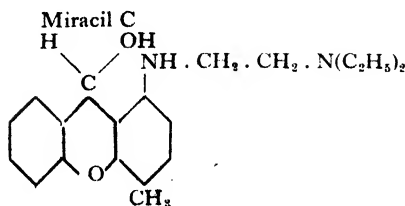
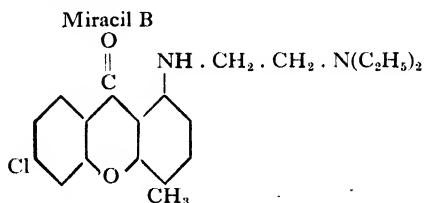
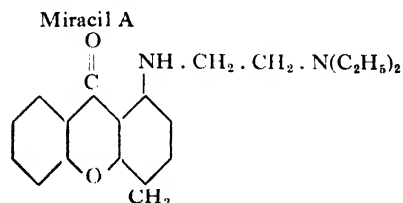
In experimental monkey schistosomiasis the activity of the miracil compounds is essentially different. In this case miracil D is the best preparation, while B produces an effect only in doses which cause vomiting. Miracil A, which is only slightly active in mouse schistosomiasis, is much more active in monkeys. Miracil C takes an intermediate position, both in mouse and in monkey tests. In the treatment of monkeys, as in that of mice, oral administration is preferable, since, as is shown by the pharmacological investigations, miracil D causes marked local irritation, and after a dose of 30 mgm. per kgm. miracil C we have seen a sterile abscess develop. In both mouse and monkey schistosomiasis (a) treatment given once or twice can effect a cure, and (b) the miracil compounds have a remarkably poor action on immature worms.

The relation between chemical constitution and therapeutic activity is interesting.

Miracil B (March, 1939) = Ms. 644 = 1-diethylaminoethylamino-4-methyl-6-chloroxanthone.

Miracil C (June, 1939) = Ms. 723 = 1-diethylaminoethylamino-4-methylxanthidrol.

Miracil D (December, 1939) = Ms. 752 = 1-diethylaminoethylamino-4-methylthioxanthone.



Miracil B is formed from miracil A by the introduction of a chlorine atom into position 6. This chemical change leads to an increase in the oral chemotherapeutic index in mice from 1:1.7 to 1:15, while it practically abolishes activity in monkey schistosomiasis. The introduction of other substituents into the 6 position, substitutions in the 2, 7 or 8 positions, and change of the basic side-chain, or of the substituent in the 4 position, all bring little advantage in comparison with miracil A and B. Only the change of the xanthone (miracil A) to xanthidrol (miracil C) shows an improvement. An increase of activity in monkey schistosomiasis is produced by the oxygen of the xanthone ring in miracil A being replaced by sulphur, to give miracil D. Substitutions in the thioxanthone group produced changes in chemotherapeutic activity similar to those produced by analogous substituents of the xanthone compounds. Among the derivatives investigated special mention should be made of 1-diethylaminoethylamino-4-methyl-6-chlorothioxanthone, which corresponds to miracil B. This substance is superior to miracil B in the treatment of mouse schistosomiasis, but, like it, is practically inactive in monkeys. Detailed chemical particulars will be given in a later publication by Mauss. Consideration of all the chemotherapeutic tests shows that miracil D is the best of the xanthone and thioxanthone compounds examined.

THE MODE OF ACTION OF THE MIRACIL COMPOUNDS

When exposed to miracil treatment the schistosomes soon lose their active motility and their normal appearance. The changes in the females are particularly noticeable: the hind part of the body takes on a cloudy milky appearance; there appear to be disturbances in the activity of the intestine, since the uniform filling of the intestine with pigment is interrupted, and parts are found almost pigment-free near places with marked accumulation of pigment; the worms diminish considerably in size; and accompanying the progressive loss of transparency there is an almost complete cessation of movement, together with a loss of elasticity, so that the worms become very fragile. In the males the changes are less pronounced both as regards the diminution in size and the increase in opacity. Even with optimum treatment the worms do not die until about 14 days after the beginning of treatment. On the other hand, egg-production is disturbed within even a few days, as is shown by the formation of eggs without egg-cells, of deformed eggs, and of conglomerations of shells and yolk-cells, as well as by the excretion of shell substance in large drops.

The details of these pathological changes can readily be recognized in fixed and stained schistosomes. In the testes, ovaries and yolk-glands there is a transient increase in the relative number of ripe cells, which passes quickly to a diminution in the number of cells. The chief points of attack of the miracil compounds seem to be the sex- and yolk-cells. Injury to the other body-cells cannot be recognized. Since, apart from the intestine, the gonads and the yolk-glands are organs with intensive metabolism, it is possible that the selective action of miracil is due less to specific organotropism than to toxic action on actively metabolizing organs. The histological findings make it unlikely that miracil acts by inhibiting mitosis. These findings and the recovery of surviving schistosomes after insufficient dosage will be described in greater detail elsewhere. It is at present sufficient to state that Vogel and Minning (1947) observed similar injury to schistosomes (*S. japonicum*) in their investigations with tartar emetic, foudadin and emetine.

The difference in action of miracil on worms of different ages, described above, can

easily be explained in view of these findings concerning the toxic action on the sex-organs. In immature worms the sex-organs are not yet active, and, judging by the poor filling of the worm's intestine with schistosome pigment, the whole metabolism is low. Consequently (a) only small amounts of miracil are taken up by immature worms, and (b) the miracil finds no suitable point of attack. Since the miracil preparations remain for a long time in the body of the host, however, the schistosomes can become mature while therapeutically active amounts of the preparation are still present in the blood. In such cases there could be injury to worms for a considerable time after a treatment which might lead to effective therapeutic action. According to this explanation, the shorter the time between the infection and the beginning of treatment the less will be the probability of radical cure. The best evidence for this conclusion is given in the protocols of miracil D and monkeys (Table VI). The dosage which destroyed mature worms had only an incomplete action on worms 25 days old, and was ineffective on worms which were four and 15 days old respectively. Further evidence is afforded by the failure of prophylactic investigations, not here recorded, with miracil A, B and D in mice.

DISCUSSION AND SUMMARY

Chemotherapeutic investigations on experimental infections of mice and monkeys with *Schistosoma mansoni*, by a technique described above, have led to the discovery of a new series of effective compounds belonging to the xanthone and thioxanthone class. These compounds, which are named miracils, have differing activities in mouse and monkey schistosomiasis. In mice, miracil B is highly effective. In monkeys, miracil D is the best and produces cure in doses of 1×20 mgm. per kgm. or 2×10 mgm. per kgm., or sometimes even with 2×5 mgm. per kgm. Since the parasite is the same both in mouse and monkey, this difference in activity can be due only to the physiological differences in the two species of animals. Since monkeys physiologically resemble man in many ways, we believe that miracil D will be the best preparation for human schistosomiasis also.

Parenteral administration of miracil D is not practicable, on account of marked irritation at the site of injection. Complete pharmacological investigation in monkeys was not possible. Miracil D exerts irritative action, as shown by vomiting and diarrhoea after large doses, but severe or fatal injury in monkeys by miracil D has not yet been observed. The loss of weight frequently observed after miracil D is believed to be due not so much to a direct injury by the drug as to an indirect injury from the death and injury of the worms through successful treatment. This is borne out by the maintenance of weight after treatment with 2×100 mgm. per kgm. miracil D by mouth in infections with worms of different ages. In cases where a therapeutic action was produced—i.e., in mature or 25-day-old worms—there was some loss of weight; when the worms were four or 15 days old, treatment was unsuccessful and the weight remained unchanged.

In animal experiments miracil D has a practical advantage over present-day preparations used for schistosomiasis in that it can be given by mouth. A second great advantage is the brevity of the treatment. In judging the effectiveness of the preparation it has to be remembered that stools do not become free from eggs until 14 days after the beginning of treatment, even when a cure has been produced.

If miracil D, which is proposed for clinical trial in view of the animal results, should prove insufficiently active, or even completely inactive, it would be desirable to test the

related compounds for their value in human therapy. In that case we would first recommend miracil B or the corresponding compound in the thioxanthone series, both of which are particularly effective in mice.

The aim of our chemotherapeutic work was discovery of a compound which could be given by mouth and would be suitable for mass treatment. This aim seems to have been accomplished in the discovery of miracil, at any rate so far as experimental infections can show. By 1941, with the discovery of miracil D, the work had progressed so far that clinical trials on human schistosomiasis could have been begun. Unfortunately, we were unable to carry out such trials, since communication with schistosome districts was interrupted by the war.

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THE ACTION OF MIRACIL IN *SCHISTOSOMA JAPONICUM* INFECTIONS IN LABORATORY ANIMALS

BY

H. VOGEL

AND

W. MINNING

(From the Hamburg Institute for Naval and Tropical Diseases)

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In the foregoing paper, Kikuth and Gönner (1948) have reported favourably on the action of miracil against *Schistosoma mansoni* in animal experiments. The action of this new drug on other species of schistosomes is also a matter of interest, and, acting upon the suggestion of Kikuth and Gönner, we have studied its effects on *S. japonicum*.

MATERIALS AND METHODS

Eleven rabbits, three hamsters (*Cricetus cricetus*) and four monkeys (*Macacus rhesus*) were exposed to cercariae of *S. japonicum* by the method described by Vogel and Minning (1947). When the animals had begun to discharge parasite ova, miracil, in aqueous solution or suspension, was administered by means of a stomach-tube. A single rabbit received subcutaneous injections. Six types of miracil, kindly supplied by Dr. Kikuth and Dr. Gönner, were tested—types A, B, C, D, Ms. 803 and Ms. 786. They were given in doses which Kikuth and Gönner had found effective against *S. mansoni* in mice and monkeys. In some of our experiments dosage approximated to or reached the toxic limit, and two rabbits, not included in the table below, died from extreme fatty degeneration of the liver soon after oral treatment with 900 mgm./kgm. miracil B and 600 mgm./kgm. miracil Ms. 786 given in three and two doses respectively at intervals of two days.

The effect of the drugs was determined by daily examination of the faeces of all 18 animals for viable ova (hatching method) and by autopsy findings in 13 of them. These 13 animals were killed after a lapse of time sufficient to prove the effect of the drug on worms and egg deposits. Worms were carefully collected from the blood-vessels and were examined for numbers, distribution and motility. The worms obtained from seven autopsies were fixed, stained with alum carmine and studied for changes in organic structure. In all autopsied animals fresh samples of the intestinal mucosa were examined microscopically, in order to obtain information as to viability of eggs in the tissues and their stages of development.

RESULTS

The main details of treatment and findings are given in the table below. All 18 animals continued to discharge viable ova. This is in conformity with the autopsy findings. Living eggs of all stages, including newly laid ova, were found in the intestinal mucosa of the 13 animals which were killed, and the mature females, with a few exceptions which are discussed below, showed numerous eggs in the uterus and often one ovum in the ootype.

In all the autopsies living male and female schistosomes were encountered in numbers which, in our experience, roughly corresponded to the intensity of the infection. The worms were normally motile and paired, and the intestine was filled with digested blood. No dead schistosomes were observed. Most of the worms were found in the mesenteric veins and had not been carried into the portal branches of the liver, as happens when their vitality is impaired by drug action.

TABLE

Test no.	Animal	Miracil type	Total dose in mgm./kgm. body-weight	No. of single doses, and duration of treatment	Hatching of miracidia observed	Days between end of treatment and autopsy	No. of living worms at autopsy	
							♂♂	♀♀
1	Rabbit	A	400	11 doses within 25 days	Till autopsy	26	25	39
2	Monkey	A	300	6 doses within 8 days	"	10*	80	92
3	Rabbit	B	790	3 doses within 6 days	"	17	12	10
4	"	C	700	7 doses within 7 days	"	25	51	51
5	Monkey	C	300	3 doses within 6 days	For 1 month after treatment	No autopsy	—	—
6	Rabbit	D	600	6 doses within 6 days	Till autopsy	52	23	18
7	"	D	600	6 doses within 6 days	For 2 months after treatment	No autopsy	—	—
8	"	D	600	6 doses within 6 days	Till autopsy	44	23	23
9	"	D	970	3 doses within 6 days	"	16	65	22
10	Hamster	D	600	6 doses within 6 days	"	24	36	44
11	"	D	600	6 doses within 6 days	"	18	10	10
12	"	D	600	6 doses within 6 days	"	24	14	43
13	Monkey	D	200	2 doses within 4 days	For 50 days after treatment	No autopsy	—	—
14	"	D	350	5 doses within 11 days	For 45 days after treatment	"	—	—
15	Rabbit	Ms. 803	600	3 doses within 5 days	For 1 month after treatment	"	—	—
16	"	Ms. 803	900	3 doses within 5 days	Till autopsy	27	31	32
17	"	Ms. 786	400	2 doses within 4 days	"	21	47	45
18	"	Ms. 786	600	3 doses within 5 days	"	20	20	16

All animals were treated by mouth, except no. 1, which received subcutaneous injections.

* Died from acute bilharziasis.

The only visible effect of the drugs which could be demonstrated concerned the reproductive organs of the worms which had been stained with alum carmine. In the males the testes contained fewer spermatogenous cells than normally, and were spongy in appearance and often definitely shrunken in size. Nevertheless, the seminal vesicles of most of the males were filled with spermatozoa. Similarly, in the females the ovaries were

smaller than normal and the aggregation of oocytes was less dense at the posterior pole of the organ. There were no changes in the male and female organs in tests no. 2 and 3; in tests no. 4 and 9 the changes were slight; they were more pronounced in test no. 16, and most developed in tests no. 17 and 18, in which miracil Ms. 786 had been given. Thirty-five females resulting from experiments 17 and 18 were stained: 30 specimens showed slight lesions, as described, and egg-production was not interfered with; in the other five the changes were more pronounced—shrinkage of the ovary was extreme, the vitellaria were almost devoid of ripe yolk-cells, and the uterus contained masses of yolk-cells and shell concretions instead of complete ova. In some instances it was seen that the worms—especially the females—were somewhat smaller than the control specimens of the same age. This reduction in size was possibly also due to the action of the drug.

The satisfactory results of the action of miracil reported by Kikuth and Gönner were obtained in mice and monkeys infected with *S. mansoni*. In our experiments against *S. japonicum* we used rabbits, hamsters and a few monkeys, and our four monkey tests can therefore be compared with those of Kikuth and Gönner. In our tests on rabbits and hamsters failure of treatment may have been due to the different class of animal used. While rabbits are inadequate hosts for *S. mansoni*, hamsters are suitable for both *S. mansoni* and *S. japonicum*. We treated two *mansoni*-infected hamsters with miracil D in exactly the same way as three *japonicum*-infected hamsters (tests no. 10, 11 and 12). Hatching of miracidia in the two hamsters infected with *S. mansoni* ceased 10 and 14 days respectively after the beginning of treatment, and at autopsy, 24 days after treatment, no living worms or eggs were found.

The experiments on hamsters and monkeys clearly demonstrate that the failure to eradicate *S. japonicum* by means of miracil was not due to the kind of host used. *S. japonicum* is obviously resistant to the miracil compounds tested.

SUMMARY

Miracil A, B, C, D, Ms. 803 and Ms. 786 were tested in animals against *Schistosoma japonicum*. The parasites were not destroyed, nor was the discharge of viable ova interrupted. Only the reproductive organs showed slight degenerative changes.

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CATCHES OF BITING DIPTERA IN UGANDA, WITH ANAESTHETIZED MONKEYS AS BAIT

BY

A. J. HADDOW

AND

G. W. A. DICK

(From the Yellow Fever Research Institute, Entebbe, Uganda*)

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INTRODUCTION

Much of the work of the Yellow Fever Research Institute has been concerned with a search for the vector or vectors which transmit yellow fever among the monkeys of forested and wooded areas in Uganda. While many localities have been studied, attention has been concentrated mainly on the heavy rain-forest of Bwamba county in the extreme west, and on secondary forest growing on the shores of Lake Victoria, near Entebbe.

In 1944 yellow fever virus was isolated from a mixed lot of 12 species of *Aedes* mosquitoes taken in uninhabited virgin forest in Bwamba. Only one of these, *Aedes (Stegomyia) africanus* Theo., seemed likely, on theoretical grounds, to be a vector of yellow fever among monkeys (Smithburn and Haddow, 1946). The subsequent discovery that this mosquito is predominantly arboreal, reaching its main concentration in the forest-canopy (Haddow, Gillett and Highton, 1947), gave support to this view, as some of the monkeys involved in the forest cycle seldom descend to ground level (Haddow, Smithburn, Mahaffy and Bugher, 1947). Consequently, in the hope of finding virus in *A. africanus* or some other arboreal mosquito, much of the field-work carried out since the 1944 isolation has consisted of mosquito catches on platforms in trees. Evidence of infection was finally obtained from a lot of 187 *A. africanus* and one *A. (S.) luteocephalus* Newst. taken in primary forest in Bwaniba in December, 1947. Of the 188 mosquitoes, only seven were taken at ground level; the remainder came from the canopy (Haddow, Smithburn, Dick *et al.*, 1948).

In these catches African mosquito-catchers served as bait. As it was desirable to confirm that *A. africanus* actually fed on monkeys in the canopy, and to obtain records of other Diptera attacking monkeys, it was decided that continuous 24-hour catches, using monkeys as bait, should be made; three such catches were conducted during 1946.

METHODS

As our supply of monkeys to be employed in the continuous 24-hour catches was limited, it was necessary to use each animal for a prolonged period. A monkey had to be completely immobilized, in order that the mosquitoes which alighted to bite might be

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picked off in tubes. We decided to narcotize or anaesthetize the animals, for binding, when applied for a prolonged period, would obviously have a deleterious effect on the monkey and would screen part of it from the mosquitoes.

We found nembutal (sodium ethylmethylbutylbarbiturate) to be ideal for our purpose. The powder was dissolved in distilled water, using 1 gm. per 61 ml. of water and applying mild heat to facilitate complete solution. Subcutaneous administration was found to be rather uncertain in its action in a first dose. Better reaction was obtained by giving the drug intravenously. In the first two experiments later doses were given by the same method, but in the third experiment it was found adequate to give the first dose intravenously and the subsequent doses subcutaneously. A dose of 0.01 gm. per kgm. body-weight was given every four hours. This was adequate for deep anaesthesia for at least 12 hours, without subsequent ill effects. Under anaesthesia of this type the monkey's temperature was found to fall rather rapidly, but it was possible to maintain the body temperature in the neighbourhood of normality (102–103° F.) by placing the animal on rubber hot-water bottles wrapped in towels and refilled about every hour.

As we have not yet found a trap which is really effective for work of this kind, the mosquitoes were caught individually in tubes by an African mosquito-catcher. While this method entailed the presence of a human being on the platform, it had the advantage that, when a mosquito bit the monkey, it could be said to have done so even in the presence of an alternative host—man. It was, in any case, necessary for a watcher to be present in order to care for the monkey. Mosquitoes which alighted on the catcher or on the platform were not collected, nor were mosquitoes which alighted on the monkey but did not bite. Only those which actually bit the monkey are included in the lists given below.

RESULTS

The first experiment was carried out in June, 1946, on a platform in ironwood forest at Mamirimiri, in Bwamba. The platform, which was in the main canopy, was built at a height of 57 feet above ground level in a large parasitic ('strangler') fig, and was surrounded by dense foliage. The forest and platforms at Mamirimiri have been described in a previous paper (Haddow, Gillett and Highton, 1947). The platform used in this case was one of those already described, rebuilt at a slightly different height.

The common white-nosed redbtail monkey of Uganda was used in the experiment. As considerable confusion exists in the nomenclature of the primates, we have found it best to adhere to a single authority (Allen, 1939), even in cases where we disagree with the names which he proposes. Allen gives the name of the species used in this experiment as *Cercopithecus nictitans mpangae* Matschie. It is perhaps worth noting that we do not believe that *C.n.mpangae* can be distinguished from *C.n.schmidti* Matschie, which inhabits the rest of the country. In our opinion the correct name of this monkey should be *Cercopithecus ascanius schmidti* Matschie. The three monkeys available were used in succession, each being anaesthetized for a period of eight hours.

The experiment was of considerable interest, for two reasons. In the first place, the only redbtail monkeys available were young ones, weighing about 1 kgm. each. The bait, therefore, was very small. In the second place, the weather was most unfavourable, a cool cloudy morning with rain in the late afternoon and evening, followed by a chilly night. Under such conditions the biting activity of *A. africanus* and of most other arboreal culicines tends to be very much reduced (Haddow and Mahaffy, *in the press*).

However, even under these unfavourable conditions, 12 mosquitoes and one gadfly bit the monkeys. The results were as follows :

<i>Anopheles (Myzomyia) gambiae</i> Giles	1
<i>Aedes (Finlaya) longipalpis</i> Grünb.	2
<i>Aedes (Stegomyia) apicoargenteus</i> Theo.	1
<i>Aedes (Stegomyia) africanus</i> Theo.	8
<i>Chrysops centurionis</i> Aust.	1
Total	13

Three of the main arboreal culicines, *A. longipalpis*, *A. apicoargenteus* and *A. africanus*, were thus represented, as well as the tabanid *C. centurionis*, the arboreal and crepuscular habits of which have been discussed elsewhere (Haddow, Gillett, Mahaffy and Highton, *in the press*). *A. africanus* formed 62 per cent. of the total catch. Only three mosquitoes belonging to this species alighted on the African catcher during the work. On the other hand, both the catcher and the writers (who spent the night at ground level, with repeated supervisory visits to the platform) were attacked throughout the night by large numbers of *A. gambiae*, which is very common at Mamirimiri, whereas only one of this species bit the monkey.

A second experiment was carried out on the same platform in July, 1946. Meanwhile, the platform had been raised from 57 to 64 feet above ground level. 'Grey' or 'grivet' monkeys, *C. aethiops centralis* Neumann, were used. Two large monkeys were available, and these were used in succession for 12 hours each. On this occasion the weather was favourable, a sunny day followed by a warm clear night. The results were as follows :

<i>Taeniorhynchus (Mansonioides) africanus</i> Theo.	8
<i>Aedes (Mucicus) grahami</i> Theo.	2
<i>Aedes (Finlaya) longipalpis</i> Grünb.	3
<i>Aedes (Finlaya) ingrami</i> Edw.	1
<i>Aedes (Stegomyia) apicoargenteus</i> Theo.	5
<i>Aedes (Stegomyia) africanus</i> Theo.	23
<i>Culex</i> sp. indet.	1
<i>Chrysops centurionis</i> Aust.	16
Total	59

Once again *A. africanus* was the commonest species, forming 39 per cent. of the total catch and 54 per cent. of the total culicines taken. It is a point of interest that the tabanid *C. centurionis* formed 27 per cent. of the catch, as we suspect that this species may be a vector of the type of filariasis which is common among wild monkeys in Uganda. We have also found that *T. africanus*, though most abundant at ground level, occurs in the forest-canopy up to an altitude of 5,000 feet in every area which we have studied in detail in Uganda. As it is known to be capable of transmitting yellow fever virus under laboratory conditions (Philip, 1930), the fact that it bites monkeys in the canopy is of added interest. *A. grahami* is a common arboreal and nocturnal mosquito. Once again it may be noted that throughout the night the catcher and the writers were persistently attacked by large numbers of *A. gambiae*, though on this occasion none bit the monkeys.

The two species of monkeys studied not only are very widely distributed in central Africa, but also, on account of their habit of raiding banana, ground-nut and sweet-potato plantations, come into closer contact with man than do most other monkeys. Apart from

African species, however, we have a particular interest in the Indian rhesus monkey (*Macaca mulatta* Zimmermann), which, because of its very marked susceptibility to yellow fever, is the species most widely used in experimental work on this disease. We have established large numbers of rhesus monkeys as sentinels on tree platforms both in Bwamba and in the Entebbe area, in the hope that they may contract yellow fever, and thus indicate a focus of virus activity in the forest-canopy. To test their suitability as sentinels, it was, consequently, important to find whether or not these monkeys were bitten by the mosquitoes in which we are interested.

An experiment was therefore carried out in secondary lake-shore forest at Zika, near Entebbe, where we have various sentinel platforms. The station concerned (Zika no. 2) has one platform in the canopy, at 52 feet, and one at understorey level, at 34 feet. We decided to catch simultaneously at both levels and on the ground below the tree, in order to learn whether the vertical distribution of mosquitoes biting monkeys was similar to that previously observed in work where human baits were employed (Haddow, Gillett and Highton, 1947). Six monkeys, two for each level, were used in rotation. Each monkey was anaesthetized for a 12-hour period. The results are shown below.

Species	Level			Total
	0 ft.	34 ft.	52 ft.	
<i>Anopheles (Anopheles) implexus</i> Theo.	2	—	—	2
<i>Hodgesia sanguinea</i> Theo.	—	1	5	6
<i>Taeniorhynchus (Coquillettidia) fuscopennatus</i> Theo.	9	2	3	14
" (<i>Mansonioides</i>) <i>africanus</i> Theo.	3	3	—	6
" " <i>uniformis</i> "	—	—	1	1
<i>Aedes (Finlaya) ingrami</i> Edw.	—	6	3	9
" (<i>Stegomyia</i>) <i>apicoargenteus</i> Theo.	1	13	7	21
" " <i>africanus</i> Theo.	—	14	18	32
<i>Eretmapodites chrysogaster</i> Graham group	1	—	—	1
<i>Culex (Culex) poicilipes</i> Theo.	2	—	—	2
Totals	18	39	37	94

Again the dominant culicine biting on the platforms was *A. africanus*. It represented 36 per cent. of the catch at 34 feet and 49 per cent. of the catch at 52 feet. Even though none was taken at the ground-level station, it formed 34 per cent. of the combined total for all levels. Though *Taeniorhynchus* spp. are particularly prevalent at Zika, they represented only 22 per cent. of the total for all levels. *A. apicoargenteus*, which showed its usual preference for the understorey level, represented 22 per cent. of the total.

The *Taeniorhynchus* spp., in an area where they form by far the most abundant group attacking man, constituted only about one-fifth of the mosquitoes taken on the monkeys. For this reason we compare below results obtained in the 24-hour catches made on man with those made on monkeys. A convenient grouping comprises *A. africanus* and *A. apicoargenteus* (the only two species taken in all three catches), *Taeniorhynchus* spp., and other Diptera. One of us (A.J.H.) has made 30 24-hour catches at Zika, five at each of the six stations so far built in that area. In each case the work was carried out simultaneously in the canopy, at understorey level, and at ground level; and in each case the bait consisted of three Africans at each level, catching on themselves. This work, which has been in

progress over a long period and in many kinds of weather, forms a good standard of comparison for the observations on monkeys. The percentage distribution of the different groups is as follows.

Catches at Zika		
Species	Human bait, per cent.	Monkey bait, per cent.
<i>A. africanus</i>	7	34
<i>A. apicoargenteus</i>	2	22
<i>Taeniorhynchus</i> spp.	78	22
Others	13	22

It may be seen that there is a very decided difference between the catches made on man and those made on monkeys. With man used as bait, *Taeniorhynchus* spp. form the bulk of the catches, while the two *Stegomyia* spp. represent only 9 per cent. In the catches on monkeys, *A. africanus* is the most abundant species taken, and the two *Stegomyia* spp. combined make up more than half of the total catch. A further note of interest is that 66 per cent. of the mosquitoes taken biting the monkeys belong to species which habitually breed in holes in trees. In the catch made on platforms, 80 per cent. of the mosquitoes taken biting monkeys belong to species breeding in tree-holes.

To date, a total of 176 24-hour catches have been made with the catchers themselves as bait, in the forest-canopy in Bwamba, at Zika and at another locality near Entebbe. Having been carried out over a prolonged period and under varied weather conditions, these catches form a good sample of the Diptera attacking man in the canopy. If we compare these with the three catches made in the canopy with monkeys as bait (i.e., experiments 1 and 2 at Mamirimiri and the catch made at 52 feet above ground at Zika), using the same groups as before, the following percentages are obtained.

Catches in the forest-canopy in Bwamba and the Entebbe area		
Species	Human bait, per cent.	Monkey bait, per cent.
<i>A. africanus</i>	19	45
<i>A. apicoargenteus</i>	4	12
<i>Taeniorhynchus</i> spp.	34	11
Others	43	32

Here again the two *Stegomyia* spp. represent more than half the total catch with monkey bait, but are very moderate (23 per cent.) in the catches made with human bait.

In addition to these facts, it may be pointed out that captive *A. africanus* feed very readily on monkeys. In one experiment (Haddow and Mahaffy, *in the press*) 3,323 mosquitoes belonging to this species were given an opportunity to feed on rhesus monkeys. Of these, 2,883, or 87 per cent., bit the monkeys, and 2,808, or 85 per cent., engorged fully.

Taking into consideration the facts that *A. africanus* attains its greatest numbers in the forest-canopy, that it bites after dark, when monkeys are asleep, that it bites monkeys

freely in the laboratory, and that it was the most abundant species in all three catches made with monkeys as bait, we may reasonably conclude that in nature this species probably feeds mainly on monkeys. This is probably also true of *A. apicoargenteus*. The latter species is, however, of minor interest where yellow fever is concerned, as it has failed to transmit virus under laboratory conditions both in West Africa (Bauer, 1928) and at Entebbe (Smithburn, private communication).

We may conclude by giving a general summary of the Diptera taken in the three catches, recording both the numbers of catches in which each species was taken and the total number taken. Totals for the three catches, irrespective of level, are as follows.

Species	No. of catches in which taken	Total no. taken
<i>Anopheles implexus</i>	1	2
" <i>gambiae</i>	1	1
<i>Hodgesia sanguinea</i>	1	6
<i>Taeniorhynchus fuscopennatus</i>	1	14 (8%)
" <i>africanus</i>	2	14 (8%)
" <i>uniformis</i>	1	1
<i>Aedes grahami</i>	1	2
" <i>longipalpis</i>	2	5
" <i>ingrami</i>	2	10 (6%)
" <i>apicoargenteus</i>	3	27 (16%)
" <i>africanus</i>	3	63 (38%)
<i>Eretmapodites chrysogaster</i>	1	1
<i>Culex poicilipes</i>	1	2
" sp. indet.	1	1
<i>Chrysops centurionis</i>	2	17 (10%)

The breeding-place of *Chrysops centurionis* is not known, but 72 per cent. of the mosquitoes taken in these catches belong to species which breed in holes in trees.

Taking the totals for catches made in the canopy only, the following figures are obtained.

Species	No. of catches in which taken	Total no. taken
<i>Anopheles gambiae</i>	1	1
<i>Hodgesia sanguinea</i>	1	1
<i>Taeniorhynchus fuscopennatus</i>	1	3
" <i>africanus</i>	1	8
" <i>uniformis</i>	1	1
<i>Aedes grahami</i>	1	2
" <i>longipalpis</i>	2	5
" <i>ingrami</i>	2	4
" <i>apicoargenteus</i>	3	13 (12%)
" <i>africanus</i>	3	49 (45%)
<i>Culex</i> sp. indet.	1	1
<i>Chrysops centurionis</i>	2	17 (16%)

In the canopy, therefore, there were only three prevalent species, *A. africanus*, *A. apicoargenteus* and *C. centurionis*. Of these, *A. africanus* was by far the most abundant. Of the mosquitoes taken, a remarkably high proportion (86 per cent.) belonged to species which breed in holes in trees,

We may note finally that, during routine work on platforms at Zika, we have observed *A. africanus* biting sentinels which had not been anaesthetized, and that in the same area we have taken *T. (C.) aurites* Theo. (a mosquito with marked arboreal tendencies, not included in the catches described above) biting rhesus monkeys after dark.

SUMMARY

1. Twenty-four-hour catches of biting Diptera, with anaesthetized monkeys as bait, were made in forest in Bwamba county and in the Entebbe area, Uganda. In two cases the catches were made in the forest-canopy only. In the first of these, redbait monkeys (*Cercopithecus nictitans mpangae* Matschie) were used, and in the second 'grey' or 'grivet' monkeys (*C. aethiops centralis* Neumann). The third catch was made simultaneously in the canopy, at understorey level and at ground level, with rhesus monkeys (*Macaca mulatta* Zimmermann) used as bait.

2. In all three catches the most abundant mosquito biting the monkeys was *Aedes (Stegomyia) africanus* Theo., a species which we know to be involved in the forest cycle of yellow fever in Uganda. Other prevalent species in the forest-canopy were *A. (S.) apicoargenteus* Theo. and the tabanid *Chrysops centurionis* Aust., which we suspect to be a vector of filariasis among Uganda monkeys.

3. It is shown that, with monkeys used as bait, *A. africanus* and, to a lesser extent, *A. apicoargenteus* form a much larger percentage of the catch than when African catchers, using themselves as bait, are employed. *Taeniorhynchus* spp. and *Anopheles (Myzomyia) gambiae* Giles appear, on the other hand, to attack man more readily than monkeys.

4. It is concluded that *A. africanus* and *A. apicoargenteus* probably feed mainly on monkeys in nature.

5. So far, we have recorded 15 species of mosquitoes and one tabanid (*C. centurionis*) biting monkeys in Uganda. Of the mosquitoes taken biting monkeys in trees an exceedingly high proportion (86 per cent.) belong to species which normally breed in tree-holes.

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THE POISONOUS WILD CLUSTER YAM, *DIOSCOREA DUMETORUM* PAX, AS A FAMINE FOOD IN THE ANGLO-EGYPTIAN SUDAN

BY

N. L. CORKILL

(From the Department of Tropical Hygiene, Liverpool School of Tropical Medicine)

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INTRODUCTION

In 1938, in the southern Fung area of the Blue Nile province of the Anglo-Egyptian Sudan, locusts did much damage to crops, and as a result famine conditions threatened. These were staved off or modified by the use of bush products, prominent amongst which was the poisonous wild cluster yam, *Dioscorea dumetorum* Pax. This yam was so widely used and played so important a part in the local diet (Corkill, 1948) that it seems appropriate to put together some information concerning it. Of the 12 or so wild roots and tubers used in the area during the famine, it takes pride of place by virtue both of the extent to which it was eaten and of its toxicity. Burkill (1939), in discussing its close relationship to *D. hispida*, remarks that, as *hispida* is the main famine yam of Asia, *dumetorum* has a similar status in Africa.

THE PLANT AND TUBER

The tubers of the yam seen in the southern Fung were small in November, rather larger in December, much bigger in January, and quite bulky in April, immediately before the vernal season. In January they bore on the integument numerous dark-coloured beads of a resinous consistency. Identification of the tuber alone proved impossible, so a specimen was planted in the garden of the Sennar hospital and was watered daily. It flowered in May, producing blue catkins, no bulbils and no fruits; the stem bore small thorns. As Burkill (1939) states that characters in the above-ground parts have not been recorded, Plate IX, showing the foliage and flower, may have some special value. From this plant the Director of the Royal Botanic Gardens, Kew, was able to have the identification established. Dalziel (1937) records that the plant twines clockwise, left to right, and that it sometimes produces an axillary tuber, i.e., a bulbil. Burkill (1939), in reviewing our knowledge of this yam, considers it to have originated with *D. hispida* of Asia from a common ancestor. He states that *dumetorum* grows close below the surface of the soil, and is thus easy to dig up, and that where it occurs it is usually abundant. He also states that in Africa the poisonous races are planted on the outside of yam-patches, in places exposed to theft, so that thieves may steal what is of little worth.

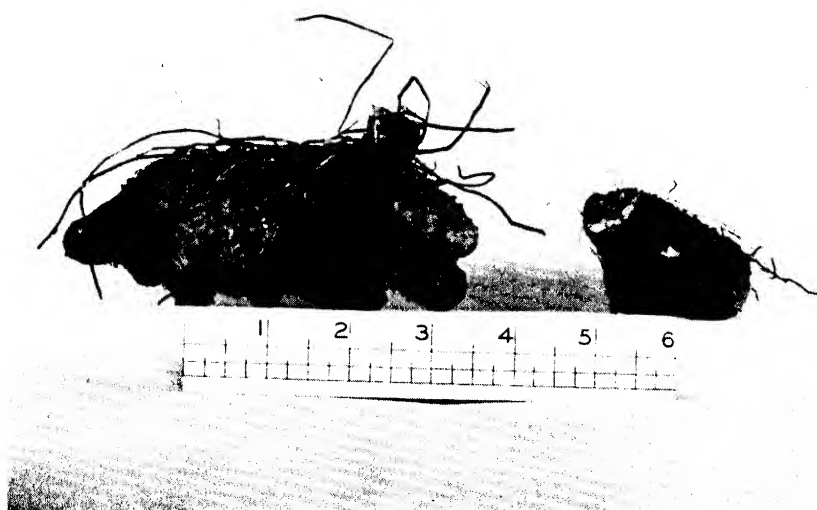
DISTRIBUTION

Burkill (1939) points out that the desert areas of Persia, Baluchistan, Sind and Arabia separate *dumetorum* from the related form *hispida* in the more humid parts of Asia. He gives the distribution of *dumetorum* as more or less 10° north where the Sahara is approached, but further northwards on the east and west in Abyssinia and Eritrea and in Upper Senegal respectively. To the south, it is found in general as far as 10° south, except for the drier



The wild cluster yam, *Dioscorea dumetorum* Pax: the flowering plant. The stem was thorned and the catkins were blue.

Courtesy of the Director, Royal Botanic Gardens, Kew.



The wild cluster yam, *Dioscorea dumetorum* Pax : the tuber.

parts of Kenya and Tanganyika. It reaches as far south as the Transvaal, and is found in both Rhodesias.

He also records the distribution of its cultivation, and specifically mentions Sierra Leone, the Gold Coast, Dahomey, the Cameroons, Chad, Ubangi-Shari, Leopoldville province, Bas-Congo subprovince, Costermansville province, Stanleyville province and Angola. These are all on the west side of the continent. Nye (1940) writes of a cultivated wild yam, *D. hispida* var. *dumetorum*, in Uganda.

In the Sudan its distribution has been given by Broun and Massey (1929) as Kassala province (Gallabat), the Nuba Mountains (Khor Ganna, near Kadugli), Bahr-el-Ghazal (Niam-Niam-land) and Mongalla (Lado district), a distribution according with Burkill's broader statement given above. The present writer has frequently encountered this yam and heard references to it in the Nuba Mountains, where it is known to both Arabs and Nuba and is commonly spoken of as a famine food, particularly in the localities of Talodi and Kau. In the Fung area likewise it is widely known as a famine food to Arabs, Ingessana, Berta, Fellata, Gummuz and Fung.

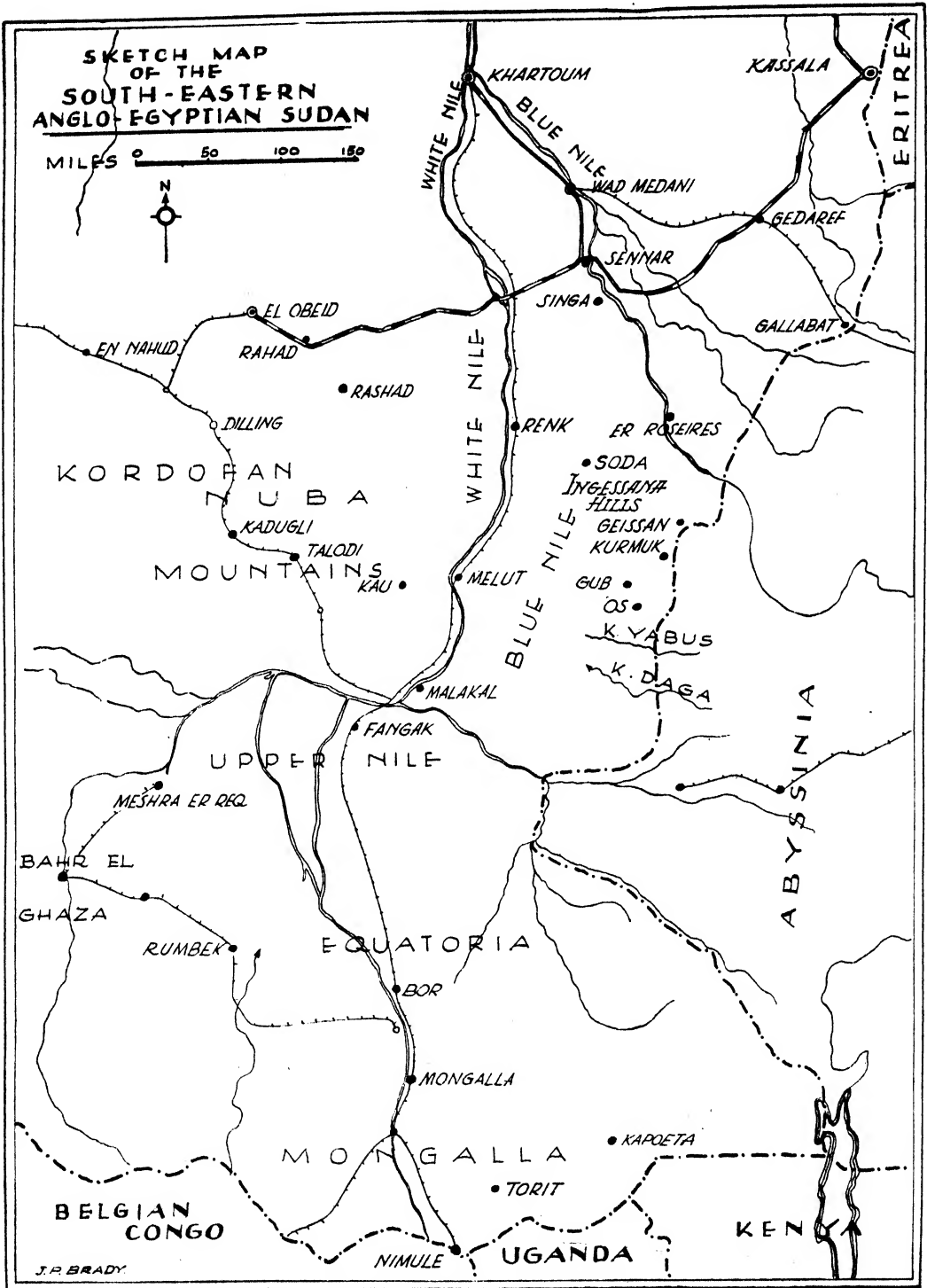
An account is given below of cases of poisoning occurring at Nimule in Equatoria after the eating of a 'wild root,' which possibly inculcates this species, and may refer to the wild yam known as the 'forest yam' to the Zande of the same province, which would seem most likely to be this species. There seems to be no record or knowledge of a cultivated form in the Sudan, unless a tuber called *rogo* at Um Ruakib near Anakhleiba in the Sennar district is one.

NAMES

Plate X shows a typical tuber of *dumetorum* and illustrates the characteristics which have given rise to certain names by which the yam is known. Dalziel (1937) gives as English names 'cluster yam' and 'bitter yam' (presumably poisonous forms only), and the present writer has heard the name 'poor man's yam' applied to the species. Dalziel also gives translations of some of the vernacular names—for instance, 'sow's teats' and 'hairy thigh.'

Vernacular names from West Africa are numerous, though the cultivated form appears to be generally known as the esuri yam. Sampson (1936), listing the yam as *D. hispida* Dennst. var. *dumetorum* (Kunth), gives the following names, apparently all from Nigeria: *bia*, *afria iwa*, *obubit iwa*, *akpana*, *edidia iwa* (Efik) and *akpana* (Ibibio). Dalziel (1937) records that in French Guinea the wild form called *budé* and the cultivated form called *bodu* are considered to be probably this species. In the Gold Coast, Irvine (1930) is quoted by Dalziel as giving the following: among the Ga, *nkamfo*, with three varieties, *akori ekyi*, *yaw serewin* and *oworo-woro*; among the Krobo, *kamfo* and *nya*; among the Ewe, *kangfo*. From Northern Nigeria are the Hausa *k'osain rogo* (in the wild state, *rogon biri* or *rogwan biri*) and (?) *gursami* (cf. below the Fellata *juzami* in the Fung), and the Tivi *inimbe*. In Southern Nigeria are the Yoruba *esuru* (varieties *fele*, *ganhun-ganhun* and *gudugudu*), the Benin and Sobo *olimehi*, *owabo* and *ufua*, the Ibo *ona*, *unu*, *adu* and *atoka*, the Efik *iwa* (generic), *afia edidia iwa*, *obubit iwa*, *ndesime iwa*, *edeminang*, *eba edi*, *iwa ekoi*, *iwa mfim* and *akpana* (which is also used for *D. cayenensis*).

In the Nuba area of the Sudan the name most commonly used by Arab and Nuba is *um bicho*, but in the Nuba tongue of Kau the yam is called *wi'urr*. In the southern Fung, Arab and Fung call it *um talga* or *um bicho*—usually the former. The Berta, however, have other names for it, *shoba* or *shatta* (cf. the Arabic for chillies). The Ingessana



hillmen of Soda call it *kau* or *kaug*, while those of Sillak call it *kaneya*. The Fellata in the Fung call it *juzami* (cf. above the *gursami* of Nigeria), and the Gummuz call it *satta* (cf. the Arabic *shatta*, chillies). As has been stated above, it is probable that this wild yam is that known to the Zande of Equatoria as *gbaranvua*, meaning 'forest yam.'

THE POISONOUS NATURE OF THE CLUSTER YAM

Broun and Massey (1929) state that the cluster yam is poisonous but can be rendered edible by pounding and washing. Sampson (1936) says that the tubers paralyse the respiratory organs and cause death by suffocation, and that a piece the size of a tennis-ball will cause death in six hours. Dalziel (1937) mentions that a bitter poisonous alkaloid (dioscorine) has been found in the similar Malayan species *D. hirsuta* Bl., but that in cultivation this is more or less eliminated. He also states that *dumetorum* is said to have caused deaths in 'E. Sudan' when eaten in famine. He quotes Watt and Breyer-Brandwijk (1932) to the effect that it is used with green mealies by the Zulus as a poison-bait to catch monkeys, which are said to become stupefied. He also quotes Meller to the effect that in Nyasaland it is used as an accessory with *Strophanthus* in arrow-poison, and states that in South Africa some tribes apply it for the local relief of pain—a point suggesting some powerful biological effect. Burkill (1939) states that the intense poisonousness of *dumetorum* protects it from destruction by wild pigs.

In the Fung no cases of yam poisoning were diagnosed during the famine, but it may be that in the village of Ulu, where yams had been eaten for eight months (Corkill, 1948), ataxia, tachycardia and vague malaise in a woman, and anorexia, abdominal distension and abdominal pain in the community as a whole, were evidence of dioscorine poisoning rather than of mild dry beri-beri, as diagnosed. The latter diagnosis appears the more likely one to the present writer (Corkill, 1948), but both conditions may have been present. There seems no doubt, however, that deaths occurred in the Fung area from the eating of inadequately detoxicated yam, and in some places—no doubt those in which deaths had recently occurred—the people became scared of their famine staple food.

On the Yabus River it was said that if the yam was eaten unprepared it would cause vomiting, diarrhoea and death in one day, and at Os the same phenomena were said to result if it was prepared without tamarind (*Tamarindus indicus*) or desert date (*Balanites aegyptiaca*). In Roseires a child was said to have died in three hours from the effects of the yam, and another who ate a small piece was reported to have rolled about and to have died quickly. One informant stated that the fatally poisoned were attacked by delirium, vomiting, vertigo, sweating and colic, and would cry out like a dog or a drunkard. Another said that an adult who had died from the poison had had vomiting and diarrhoea, with deafness after four days, and had died on the seventh day.

These stories, by their circumstantial nature, would seem to be worthy of credence, and, as will be seen below, appear to be supported by different versions of the symptoms of *dumetorum* poisoning and by what is known from elsewhere of its nature and of the nature of dioscorine poisoning.

In Ulu it was said that, if the yam was inadequately prepared, it would seize a person by the throat. It was also said to cause abdominal pain and distension if eaten over a long period. In Roseires one informant stated that it caused salivation, lachrymation and delirium, and another that it caused vomiting, speechlessness, salivation and a sensation of heat. At Gub a poisoned person was described as foaming at the mouth, breathing heavily, weeping, having bulging eyes, suffering from a burning pain in the mouth, and

producing a yellow vomit (bile?). In Kurmuk it was said that the symptoms of poisoning were salivation, lachrymation and delirium.

Putting these opinions or experiences together, it appears that in persons eating cluster yams containing the poisonous principle death may occur in from three hours to seven days, and that symptoms may be (a) those of local effect, i.e., a burning pain in the mouth ('seizing by the throat'), inability to speak normally or deprivation of speech, abdominal pain (colic and child rolling about), vomiting—possibly of bile—and diarrhoea, and (b) those of systemic and central effect, i.e., delirium, vertigo, sweating, salivation ('foaming at the mouth'), lachrymation ('weeping'), 'heavy breathing,' sensation of heat, bulging eyes and deafness. In those eating the tuber over a long period it may be that anorexia, abdominal pain and intestinal distention may result.

From this evidence there seems little doubt that poisoning was being experienced, though, as Henry (1939) has pointed out, so far the poisonous alkaloid has not been chemically demonstrated in *dumetorum* itself. He gives the formula of the alkaloid dioscorine as $C_{13}H_{19}O_2N$. It was first obtained by Boorsma (1894) from *D. hirsuta* and later by Leyva and Gutierrez (1937) from *D. hispida*. Henry states that dioscorine is bitter and poisonous, produces paralysis of the central nervous system, and in general behaves like picrotoxin, the symptoms of which, as given by Smith and Cook (1928), are a burning pain in the gullet, which passes into the abdomen, salivation, nausea, vomiting, deepened and quickened respiration, slowing of the pulse, vertigo, confusion, drowsiness, coma, tonic convulsions succeeded by those of a clonic nature, and death from medullary paralysis. Smith and Cook mention that milder cases may reel and stumble about and talk like drunken men. Apart from the convulsions, this picture is similar to that of the Fung composite described above.

Some points concerning poisoning by other yams are of interest. It has been said (reference untraced) that the juice of yam tubers in general has a numbing effect on the skin, and we have mentioned above the use in South Africa of an application of *dumetorum* for the local relief of pain. As symptoms of poisoning from eating inadequately prepared *D. triphylla* (= *D. hispida*), Gimlette quotes Greshoff (1893) as giving headache, cramp in the stomach, vertigo, vomiting, paralysis and, in severe cases, death. Gimlette also describes a case in Malaya of suspected acute yam poisoning with *D. triphylla* (= *D. hispida*) (though the possibility of mixture with *datura* was not ruled out), in which the symptoms were an earthy taste, stupefaction, paralysis of the legs, vertigo, parched throat and ataxia in the first 12 hours, followed by dilated pupils inactive to light, diarrhoea, abdominal distension, thirst, pain at the angles of the jaws, and inability to rise. Prain and Burkill (1936) state that in India *D. hispida*, the Asiatic counterpart of *dumetorum*, is called *marapashpoli*, a Sanskrit name meaning 'deadly stranglecake.' They also state that the Bhils use it as a tiger-poison. They quote Dymock, Warden and Hooper (1893) to the effect that a piece the size of an apple will cause intense irritation of the mouth and throat, a sense of suffocation, drowsiness, exhaustion and death in six hours; it also appears that a man may be violent in the early stages of the poisoning. In addition, Prain and Burkill (1936) record the use of *hispida* as a dart-poison adjunct in Malaya, an arrow-poison in Sikkim, a fish-poison in Java and a fowl-poison in Bali. They also record its use in folk medicine in yaws, leprosy and myiasis, and the addition of the juice of the tuber to liquor in India to produce thirst.

Reference is made below to a practice in Bali in which a related tuber is considered

to have been inadequately detoxicated if it 'writhes' in drying. One of the characteristics of the sliced *dumetorum* material obtained from Ulu was that it was twisted and curled when dried (or drying), which suggests that, if the writhing really does signify inadequate detoxication, then further support is found for the suggestion that the Ulu community mentioned above was suffering from mild dioscorine poisoning. However, whether or not this was the case, it would appear that dioscorine poisoning did occur in the Fung as a result of eating inadequately detoxicated cluster yam.

COLLECTION AND PREPARATION OF THE CLUSTER YAM AS A FOOD

Broun and Massey (1929) state that *D. dumetorum* can be rendered edible by pounding and washing. Dalziel (1937) describes it as being 'generally not used or only in scarcity, requiring to be sliced and steeped long before use.' Writing of the alkaloid dioscorine in *hispidula*, Burkill (1939) notes that, being soluble in water, it is removed by killing the cells which hold it and by exposing them to washing in water. He mentions the processes used to this end—boiling, peeling, slicing, pulping, pounding, and steeping in water (running, if possible, and also, if possible, containing salt). He states further that these ways of making an innocuous starchy meal are recorded from Africa also in the case of *dumetorum*.

In the Fung, the tuber is not cultivated (except possibly at Um Ruakib, as noted above), but it is known to have been collected from December to May. Nothing was learnt of any seasonal variation in toxicity, but it was noted that some specimens collected in January bore on the exterior of the tuber numerous beads of a resin-like exudate, which may be of interest in this connection. At the village of Gub it was said that the correct time to collect the tubers was in the *rashash* (i.e., the early rains—that is, May), when the leaves of the plant have fallen off. (Incidentally, it may be mentioned that the leaves themselves do not appear to be eaten.) In Roseires it was said that the tuber might be gathered at any time. Generally, it was collected in the bush by the women or carried in baskets from even further afield. After preparation it was eaten as a cooked whole vegetable in the form called *balila* or was ground up as a flour and eaten either as a porridge, called *asida*, or as an unleavened bread, called *kisra*. On the Yabus River it was eaten with sesame-seed as a porridge. In the village of Sillak it was used as the basis for a beer (*marisa*), apparently without any special detoxication process. Usually, however, some three days were given to processes aiming at detoxication, which varied in their nature in different places.

In Ulu, for instance, it was peeled and sliced; it was then steeped in cold water for two days and then in hot water for a further day. Alternatively, it was steeped in cold water and a soil called *bardob* (black cotton-soil) for three days, after which it was dried by exposure to the sun and then ground to a flour on the stone rubbing-quern (*murhaka*). It was then made into *kisra*. Several methods were in use in the Roseires district. In one method it was put in the wet sand of a water-course for a day, then peeled and sliced and placed in water containing the ash locally called *kumbo* from plant stalks for a second day, and finally soaked in cold water for a third day and then prepared as a *balila* or *kisra*. Another method was to peel and slice and then to mix with sand for a day, followed by soaking with plant-ash salts for a day, and by a final soaking for a day in cold water. A third Roseires method was to heat the tuber in hot water for some hours and then to slice and peel it and finally to steep it in a mixture of water and cotton-soil for three days. On

the Yabus River it was cooked in water for about two hours, then peeled and sliced and put to steep in hot water mixed with plant-ash salts for about 6–8 hours, and finally squeezed in the hands to remove surplus water. In Sillak the villagers said that they steeped it for three days and then cooked it as *balila*. In Kurmuk it was first peeled and then put in a pot of water on the fire and boiled. It was then sliced and steeped in water, which was changed daily until its taste denoted that all poison had been removed—a procedure taking two or three days. The Jum-Jum method was to peel, put in cold water for two days and then in hot water for one day. In Geissan the tubers were put in wet sand for several days. In the village of Os and in several other places nearby, the necessity was stressed of preparing (i.e., cooking) the tuber, either with a tamarind mass (called *aradeib*) or with the desert date (*lалob*). The pods of the tree *T. indicus* (the pods and the tree are both called *aradeib*) are marketed widely in the Sudan as a confection—or, rather, an agglomerated mass—and are used as a household remedy, a laxative, and the means for making a refreshing drink. Their use as a detoxicator is probably inspired by magic, i.e., they are believed to purge (*sic*) the tuber of its poison. The desert date is characteristically emetic as well as purgative, like the tamarind pod, and its reputed value as a detoxicator would also appear to be inspired by magic, in that it is cathartic. There is possibly some reinforcement for these superstitions in the fact that, as the root causes vomiting, abdominal pain and purgation, so a substance with a similar action may cure or prevent these conditions—*similia similibus curantur*. The yam was first boiled in a pot for two hours with either of these two adjuvants, then peeled, sliced and mixed with hot water and plant-ash, or with the salts (called *kumbo*) crystallized from it, for 6–8 hours, and finally washed in clean water and squeezed to remove surplus moisture. It was noted at Ulu that the finally prepared slices of the yam, which were tasteless to the present writer, were twisted and curled.

Prain and Burkill (1936) give modes of preparation and tests for the elimination of the poison from the related form, *hispida*, as practised in the East Indies. All appear to involve gross physical decomposition by cutting or pounding, by soaking, by treatment with salts, and by washing. In Bali the preparer is not satisfied if the slices writhe when drying (see above for the warping and twisting of prepared *dumetorum* at Ulu). Should any doubt exist, some of the slices are given to a fowl, and, if it shows signs of dizziness, preparation is considered incomplete. Elsewhere, a drop of fluid from the prepared mass is dropped into the eye; if the eye smarts inadequate preparation is indicated. On the other hand, if shrimps or fish gather round the basket in which the tuber is being steeped in running water, then the poison is considered to have been washed out.

NUTRITIVE VALUE

Table I shows the amounts of certain nutrients obtained from *D. dumetorum* by the Government Chemist, Khartoum, together with the total calorific value.

As the Ulu people showed no signs of scurvy after eight months on a diet mainly of yam and guinea-fowl, the writer, for lack of a better means, assessed the presumed ascorbin value by the decolorizing effect of the tuber on dichlorophenolindophenol. The results are shown in Table II and suggest that the tuber may have a very useful content in ascorbin. The tubers were dried, peeled, cut up and then pounded to a mash with water in quantities of 20 gm. The values are expressed in mgm. per 100 gm. of peeled tuber. The values are those given by simple titration with dichlorophenolindophenol, no other reagents

TABLE I

Some nutrient values for the wild cluster yam, *D. dumetorum* Pax, in the southern Fung, Sudan

Percentage of waste (integument)	Values per 100 gm. of edible portion					
	Total calories	Protein	Fat	Carbohydrate	Calcium	Ascorbin
10	129	4.2 gm.	0.4 gm.	26.3 gm.	92 mgm.	6.6 mgm.*

* This value was obtained by titrating peeled raw tubers pounded up in water against dichlorophenolindophenol dye. The presence of interfering substances was not allowed for, so the value may not be a valid one.

being used. They are therefore possibly invalidated by the presence of interfering substances. As the mode of preparation and the amount of cooking varied widely, a value of 2 mgm. per peeled tuber may perhaps be used as a tentative working value in calculating the value in a diet. Cooking in water was found to increase the weight of the tuber by 8 per cent. A piece of local fresh tamarind cake was tested in Roseires in February for presumed ascorbin content by titration with dichlorophenolindophenol, the material being well diluted to secure colour appreciation; a value of 100 mgm. per 100 gm. was obtained. Preparation with tamarind may thus be expected to increase the ascorbin content of the yam.

DISCUSSION

Dr. R. Kirk, of the Sudan Medical Service, has informed the writer of the occurrence in 1940—a time of severe famine—of a number of cases of what appeared quite possibly to be yam poisoning in a village some 20 miles south of Nimule in Equatoria. The details were originally supplied to him by Dr. K. Malone, S.M.S. Seven out of eight persons

TABLE II

Assumed ascorbin values* for the cluster yam under various conditions in the southern Fung, Sudan

Place	Date	State	Value (mgm. per 100 gm.)	Remarks
Ulu ...	Jan., 1939	Big tubers, 3 weeks old, unprepared, peeled, uncooked	6.6	Tuber probably approaching mature storage phase
Roseires	Apr., 1939	Big tubers, 1 week old, peeled and prepared by 3 days' steeping and washing, uncooked	6.6	Probably mature
Kurmuk	Nov., 1939	Very small tubers, 1 week old, peeled, unprepared, uncooked	2.2	Immature
Wisko	Dec., 1939	Medium-sized tubers, 1 week old, peeled, prepared with tamarind, cooked as a solid	30.0	<i>Tamarindus indicus</i> † may have contributed most of the value
Roseires	Jan., 1940	Big tubers, 1 week old, peeled, prepared with tamarind, uncooked slices	8.0	See above
Roseires	Jan., 1940	Sample of preceding specimens, after 8 hours' cooking	1.0	

* For lack of better facilities, the yams were titrated against dichlorophenolindophenol for decolorization, and the result was tentatively assumed to indicate the quantity of ascorbin present. The presence of interfering substances may have distorted the result.

† The remarks in note* above apply also to an estimation of the ascorbin value of tamarind carried out by the writer, which gave a value of 100 mgm. per 100 gm. Fixsen and Roscoe (1940) give an ascorbin content of 41.8 mgm. per 100 gm.

ate from a common bowl of food said to have contained cooked wild root. The symptoms were sudden onset of weakness, loss of vision, vomiting and inability to swallow. Six of the persons died within 3–4 days, and the single recovery is known to have been ill for several weeks. Diagnosis was not finally established, and, although the cases were noted to have features resembling botulism, yam poisoning was considered a possible diagnosis.

In botulism the toxic effects are due to the poison produced by the bacillus in its anaerobic growth in preserved food, both meat and vegetable, and, although the bacillus itself exists in virgin soils, poisoning is due to the ingestion of preserved food containing the toxin, and not of the bacillus itself. Before the development of symptoms there is a latent period of between 12 and 48 hours, and death usually occurs in 1–2 weeks. The symptoms are headache, vertigo, drooping of the lids, double vision, dilated pupils, and paralysis of the muscles of the tongue, larynx and throat, which cause inability to speak or swallow. There may be nausea and vomiting, especially in fatal cases. There is abdominal distension and constipation, and most secretions are inhibited or diminished. From the data available on the cases of poisoning, it is difficult to make a certain diagnosis on clinical grounds, but the facts (a) that all the cases had eaten a wild root in famine time (which suggests that the root and its preparation were perhaps not well known to them), and (b) that *D. dumetorum* almost certainly occurs in the area, would seem to point to the yam as having been the cause of the catastrophe.

The phenomenon of ennoblement is of some interest. It has been noted above that on cultivation the tuber tends to become free from poison. The mechanism would seem to be simple selection—that is, the species varies naturally in its dioscorine content, which contributes the attribute of bitterness as well as of toxicity, and so, as man rejects the bitter forms and replants the less bitter, the cultivated type becomes prevaillingly innocuous, while, in the bush, wild pig—and perhaps monkeys—eat the less bitter and leave the more bitter to propagate more preponderantly. Gimlette (1929) has remarked that in Malaya it is the mature form of *D. triphylla* (= *D. hispida*) that is eaten, the less mature being the more poisonous. It may be recalled that the young specimens collected in January in the Fung bore on the integument beads of a resinous exudate. It would be interesting to know if these beads are a protective structure heavily charged with alkaloid, the object of which is to prevent their being eaten by wild game before the plant has reached maturity.

SUMMARY

1. Following a locust visitation which destroyed much of the crops in the Fung area of the Sudan in 1938, some 12 wild tubers and roots were widely used as food.
2. The most important was the cluster yam, *Dioscorea dumetorum* Pax, presumed to contain the alkaloid dioscorine.
3. Cases of poisoning resulted from its use as a food, although most communities soon evolved methods of detoxication.
4. The yam was usually prepared either as a cooked whole vegetable, as a flour for making cakes, or as a porridge. Beer was also made from it.
5. Its nutritive value was mainly as a calorie food, but it also contributed most protein to the famine diet, and seems to have been the main antiscorbutic food, being thus analogous to the potato in parts of Ireland.
6. Two closely related forms from Asia are referred to.

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RELAPSING FEVER IN CYPRUS

BY

R. M. GAMBLES

(Veterinary Research Officer, Government of Cyprus)

AND

N. F. COGHILL

(Physician, West Middlesex Hospital; Late Major, R.A.M.C., Medical Specialist)

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INTRODUCTION

No evidence has been found of any case of relapsing fever in Cyprus diagnosed microscopically prior to 1939, though there is some evidence that the disease has for some time been of sporadic but wide-spread occurrence in rural districts throughout the island. The only reference to Cyprus in this connection that can be found before 1943 is in a paper by Carlisle (1906), who mentions the island (on what evidence is not known) as an occasional source of 'bilious typhoid,' a condition originally shown by Griesinger in Egypt to be louse-borne relapsing fever. The island is not mentioned by Burnett (1816), who describes what is presumably louse-borne relapsing fever in the Mediterranean area.

We are indebted to Mr. Theophilus Mogabgab, Antiquities Officer at Famagusta, and to the Director of Antiquities to the Cyprus Government for the following information. In 1935, during excavations in the underground passages of the mediaeval fortifications at Famagusta, about 20 of the workmen were at different times bitten by 'insects.' These 'insects' were numerous, and some were seen in the act of biting—usually on ankles and legs. There were large numbers of bats and rats in the galleries, on which the insects, thought to have been ticks, may normally have fed. The Landgate, where most of the subsequent cases of fever occurred, also harboured 'flying foxes' (large fruit-eating bats). The galleries when opened up contained very considerable quantities of guano (up to 1 foot deep), probably mostly derived from bats. The men who had been bitten became ill with fever which recurred every 4-7 days and usually lasted 1-3 days. Several became jaundiced, usually after they had been ill for about a month or more. Their illness lasted from one to six weeks and in one case for a much longer period. There were no deaths. The cases, probably of relapsing fever, occurred among men working in at least six different parts of the old fortifications, which are very extensive. As a result of this illness certain precautions were taken—chiefly removal of the guano and a liberal use of carbolic acid solution poured over ground and walls near the workmen—after which there were no more cases in the fortifications for some years, though additional cases occurred among workmen in old underground quarries outside the walls of Famagusta.

In 1938 Mr. Mogabgab, at the age of 52, himself contracted the disease, developing his first attack of fever on September 21st. The known dates of his relapses were September 28th, October 3rd, October 12th and (probably) October 20th, and there were almost certainly subsequent milder ones. His illness appeared to him to be very similar to that of his workmen in 1935. Symptoms accompanying the fever were: shivering; severe headache (often occipital and nuchal); generalized aches and pains, particularly severe around the waist and worst on the left side; very considerable malaise and sweating; occasional nausea. He was almost completely well between the paroxysms. About a

month after the onset he became moderately jaundiced. Towards the end of the illness the bouts of fever became less severe, and the disease wore itself out with recurring joint-pains. After the illness his hair went rapidly grey and he felt weak in the knees for a long time. In this case a clinical diagnosis of relapsing fever was eventually made by the District Medical Officer of Famagusta, Dr. H. Macfie, who assumed that it was of the louse-borne type. None of the cases so far mentioned received any specific treatment.

According to Mr. Mogabgab, his workmen stated that the condition was common in the Carpas peninsula and surrounding countryside, and that they realized that it was contracted by entering 'caves,' etc., where sheep and cattle were kept. He himself has come to believe that the disease is especially associated with caves and other sites where there is organic matter or dung of sheep or bats.

Towards the end of July, 1939, about 50 miners at the Kalavassos gold and pyrites mine went sick with fever over a period of about 15 days. They were under the care of Dr. G. Christopoulos, who has kindly supplied us with this information. Without examination of the blood all received treatment for malaria. In all cases the temperature fell, but in five or six a further attack of fever developed about five days later. A blood film was taken from each of these cases and spirochaetes* were found in two of them. No treatment was given and nothing is known of their further history. The miners were not lousy. The majority lived in Kalavassos and the surrounding villages; a few lived in huts near the mine.

The first case to be reported officially to the medical authorities in which a diagnosis of relapsing fever was made by the discovery of the spirochaete in blood films was from Galata in the Solea area on September 30th, 1939. As a result, the Director of Medical Services (Civil) made the condition notifiable. Dr. C. L. Frangeskides at once reported a case which he had diagnosed microscopically early in September, 1939, from a patient whom he attended at Limassol.

The next civilian case to be reported occurred in December, 1941, in a six-year-old girl from a Nicosia family who had been evacuated because of the war to a village (Milia) in the Famagusta district. After living there for six weeks the child became ill, and spirochaetes were found in the peripheral blood. The patient was not lousy. The health officer investigating the case found numerous argasid ticks in the cracks and holes of a mud wall, where fowls were perching at night, a few feet away from the room where the child slept. A number of these ticks were identified by one of us (R.M.G.) as *Argas persicus*, an almost invariable inhabitant of poultry-houses in Cyprus. No other investigations were made in this case.

At that time no species of *Ornithodoros* had been recorded from Cyprus, the only argasid tick known to be present being *A. persicus* Oken. (An early record by Williamson (1908) of *A. reflexus* as a parasite of fowls transmitting avian spirochaetosis almost certainly refers to this species.) As has happened elsewhere, *A. persicus* was at first thought to be the transmitting agent in the cases of relapsing fever which had occurred, but no sound evidence has ever been produced that this tick can transmit spirochaetosis to humans. Lice were next considered as vectors, but no evidence was forthcoming to incriminate them.

* In describing the parasite responsible for relapsing fever in man we have retained the term *Spirochaeta*. Other names, principally *Borrelia*, have been used from time to time to designate the organism, but, as pointed out by Strong (1945), in the present state of knowledge it is better to retain the original well-known term. This matter is discussed, in the same vein, by Hindle (1931).

From August, 1941, cases of relapsing fever began to occur among troops stationed on the island, mostly after recent contact with caves. Ninety-eight cases, in seven of whom blood films were negative, were reported between August, 1941, and April, 1943. Table I, which includes the cases of Wood and Dixon (1945), shows the monthly incidence, which was dependent on such factors as the size of the garrison, the holding of manoeuvres and the time of year. Although there were several variables, the fact that the maximum incidence in 1942 was from February to July is against the louse being the transmitting agent.

TABLE I

Monthly incidence of relapsing fever cases in the army of Cyprus

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1941								1	2	—	1	1
1942	2	10	10	2	15	20	8	5	—	3	9	2
1943	—	6	—	1								

TOTAL 98

Tables II and III give the site of infection in 74 cases (including four without positive blood film) where it is known with relative accuracy. The cases of Wood and Dixon (1945) are also included in these tables. As will be seen from the accompanying map, the distribution of the disease was wide-spread throughout the island.

Another civilian case diagnosed by positive blood film occurred in November, 1943, from near the 57th General Hospital site at Nicosia.

We have no records of military cases after April, 1943, although some are known to have occurred. Further clinical civilian cases appeared in 1945 at Sotira (site A, VII; see below), where ticks were found and identified.

Altogether 28 military cases (including six reported by Wood and Dixon) occurred in sites where ticks were subsequently found. To these may be added the civilian case in November, 1943, and the clinical cases at Sotira in 1945.

The increasing incidence among troops led to a more detailed search by the military authorities for transmitting agents. As with the civilian cases, lice were suspected for a time; but, although one of the affected Indian units was known to be at least 40 per cent. lousy, and many of the earlier Indian patients were infested, it became clear fairly soon that few, if any, of the cases were louse-borne, since the disease occurred in personnel who were not lousy and who could not have had any recent contact with lice, and since it continued to occur in units after adequate delousing. Furthermore, the clinical course of the disease did not suggest the louse-borne type. *Ornithodoros* ticks were eventually found where lousy patients had contracted relapsing fever.

In March, 1942, three military cases occurred at Kokkinitrimithia, 9½ miles west of Nicosia. (These are among those reported by Wood and Dixon, 1945.) The men had all slept in a cave 8–9 days before contracting the disease. As reported by Wood and Dixon, search of the cave revealed a few ticks, which were identified by Professor Adler, of the Hebrew University, Jerusalem, as *Ornithodoros tholozani* (Laboulbène and Mégnin) (syn. *papillipes* Birula). Similar ticks from an adjoining cave collected soon afterwards by one of us (R.M.G.) were also forwarded to Professor Adler, who stated that they were the same species.

TABLE II
Site of infection in 59 cases of relapsing fever from relatively static army units

Site	No. of cases
57th General Hospital, Nicosia (site A, VI)	1*
Kokkinitrimithia (site A, I)	6*
Episkopi (site A, IV)	12*
Erimi (site A, III)	3*
Ktima (Paphos), north-east corner of town (site A, V)	4*
" " other parts (site B, IV)	4
Limassol aerodrome (site C)	6
Lefka (site A, II)	2*
Near Lefka (site C)	1
Near Nicosia (site C)	1
Yeroskipos (site B, III)	1
Larnaca area (site B, II)	3
Lakatamia (site C)	1
Kormakiti (site C)	1
Dekelia (57th General Hospital) (site C)†	1
Famagusta old fortifications (site B, I)	5
Vatili (site C)	2
Angastina (site C)	3
Kyrenia Pass (site C)	2
TOTAL	59

* Ticks subsequently found at these sites.

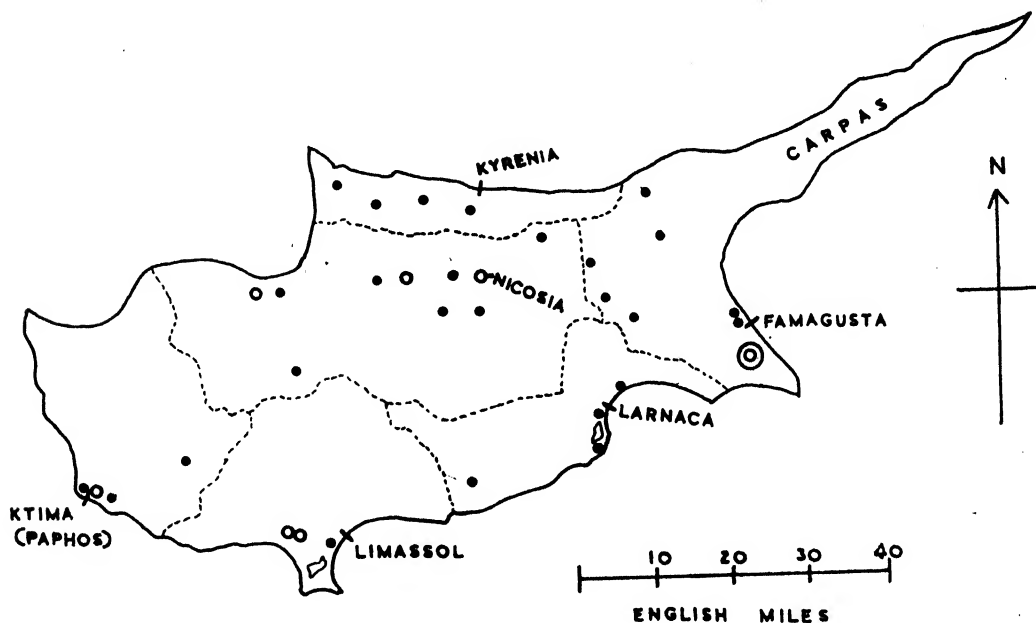
† Site of hospital before it was moved to Nicosia in 1942.

A case investigated a little later was that of a Cypriot soldier (case 18 of Coghill, *in the press*) stationed near Lefka (site A, II; see below). One of us (R.M.G.) examined the place where he had been working and the small cave where he had sat to eat his lunch, about 10 yards from the trench which he was digging. Ten specimens of *O. tholozani* were found in the cave at various stages of development. Case 10 reported by Wood and Dixon (1945) was working fairly near to this patient. One of the ticks (an adult female) was sent alive to Professor Adler, who informs us that, after feeding on a guinea-pig without infecting it, the tick laid a batch of eggs. Larvae hatching from these eggs, when fed on another guinea-pig, infected it with relapsing fever spirochaetes. It is not unusual for adult ticks to fail in transmitting spirochaetes, even though they are ultimately shown to be infected, the reason being that at the time of feeding the tick has no spirochaetes in the

TABLE III
Sites where 15 army cases of relapsing fever were infected on manoeuvres
(all site C)

Site	No. of cases
Platres (Paphos area)	4
Nicosia (4 miles west)	1
Kyrenia (Myrtou area)	2
Lefkoniko area	1
Kondea area	2
Kalokhorio	1
Famagusta quarries	1
Akaki area	2
Unknown	1
TOTAL	15

salivary glands; although they are present in other parts (Adler, Theodor and Schieber, 1937; Slavina, 1944). As is pointed out by Adler *et al.* (1937), writing of *O. tholozani* in Palestine, for the same reason, or because a number of eggs from infected mothers may be clean, many individual larvae from such mothers are incapable of transmitting the disease. Further, as Davis (1941) has noted, the infectivity of individual ticks varies from bite to bite. And there appears to be some evidence for supposing that larvae and nymphs are often more infective than adults. Professor Adler's successful transmission-experiment may be considered satisfactory proof that relapsing fever in Cyprus can be transmitted by *O. tholozani*, which is sometimes naturally infected.



MAP of Cyprus, showing district towns and boundaries.

- Sites where cases of relapsing fever occurred.
- Sites where *Ornithodoros tholozani* was found following the occurrence of relapsing fever.
- ⊙ Site where *Ornithodoros tholozani* was found following the occurrence of unproved cases of relapsing fever.

Dixon (1943) has presented additional evidence of the tick-borne nature of relapsing fever in Cyprus by showing that guinea-pigs can be infected with the responsible spirochaete taken from a patient with the disease.

At the end of their paper Wood and Dixon (1945) state that the spirochaete causing the disease in Cyprus is *Spirochaeta hispanica*, not *Sp. persica*. Their only reason for this supposition appears to be that the spirochaetes were infective for guinea-pigs; but this is not sufficient ground for such belief, as will be apparent from a previous discussion of the subject by Coghill and Gambles (1948), and from the fact that Nicolle and Anderson (1928) and others have found *Sp. persica* markedly pathogenic for guinea-pigs. Although at present there is no actual evidence, it seems best on geographical considerations and on those of vector specificity to postulate that the relapsing fever with which we are dealing here is caused by the same tick-borne parasite as in Palestine, namely, *Sp. persica* (syn.

sogdianum). However, the concept of 'vector specificity' and 'species unity' of spirochaetes elaborated by Davis (1942) is by no means of universal application, as is shown by Nicolle and Anderson (1928) and by Brumpt (1939), and discussed by Hindle (1931), who quotes further work of Nicolle *et al.* on the subject.

ENTOMOLOGICAL OBSERVATIONS

Circumstances did not permit the authors to search all sites where cases occurred. Of those examined with negative results, most were difficult to investigate, either for structural reasons or because exact movements of the patients were not known. Wood and Dixon, in a personal communication, have given us the locations of sites A-F described in their paper of 1945. These sites are, where appropriate, referred to in brackets below. Our own cases are those reported by Coghill (*in the press*).

A. SITES WHERE TICKS WERE FOUND

I (a). *Kokkinitrimithia*. ('Cave A' of Wood and Dixon, 1945.) Three of the cases reported by Wood and Dixon occurred on this site, where the first ticks were found on April 9th, 1942. The cave was situated in a depression in the ground north of the village, where quarrying operations had at one time been carried out on a small scale. At the edge of this depression, under the lip of a low cliff bounding it, were several large hollows. The roomiest of these had been enlarged to form a cave and was reinforced with sandbags, which screened the mouth and extended along one of the inner walls. It was here that Majors Clark, Wood and Dixon first found the ticks. It was revisited on July 22nd, 1942, by one of us (R.M.G.), but no further ticks could be found. The largest of the hollows left in its natural condition, situated a few yards from the sandbagged position, was three or four feet high, about 15 feet wide, and at its deepest part extended backwards for about 12 feet. Samples of sand and earth were collected from various parts of the floor of this second 'cave' and examined carefully by spreading out on a smooth black surface. All samples from the mouth of a small hole in the wall near the back, which might have been a rat-hole, contained a few ticks at various stages of development. There was no direct evidence that the hole was that of a rat, but the quarry had been used as a general rubbish-dump by the village and was the type of place where rats might be expected to abound.

I (b). *Kokkinitrimithia*. ('Cave D'.) Two of the cases reported by Wood and Dixon (1945) and one of the present authors' occurred on this site, which was a cave a quarter of a mile west of the village, behind a cook-house building where two of the cases had been working and sleeping. It was a wide and fairly low cave, with a sandy floor, open back and front, the back leading to a depression in the ground five or six yards over a low ridge, in the face of which the front of the cave was situated. It was visited by Wood, Dixon and one of us (R.M.G.) on September 30th, 1942. There were many rodent-tracks along the sides of the cave, but the only hole found was full of cobwebs and obviously no longer inhabited. Samples of earth from the floor of this hole were obtained by means of a dessert-spoon strapped firmly to a stick and were shaken through a 20-mesh wire sieve. These and other samples of sand and dust from the floor of the cave produced no ticks. Major Dixon, however, obtained a single specimen of *Ornithodoros* by sieving scrapings from the roof. A rat-trap was set, with a view to examining wild rodents from infected sites, to see if they were acting as reservoirs of infection. A 'spiny-mouse' (probably

Acomys dimidiatus nesiotus, the only species so far recorded from Cyprus) was caught in the trap, but no spirochaetes were found in blood smears. It was later learnt that the animal's blood and emulsified brain should have been injected into a guinea-pig or rat.

II. *Lefka*. ('Cave E'). A camp about a mile and a half to the west of Lefka was visited by one of us (R.M.G.) on August 13th, 1942. There had recently been two cases of relapsing fever (our case 18 and Wood and Dixon's case 10) in a Cypriot unit, the men of which were sleeping in tents in a field where there were no rocks or caves of any sort. Inquiries revealed, however, that they had been working on a rocky hill-side nearby, and the site was inspected in company with Major Clark. About 10 yards further up the hill-side from the trench where case 18 had been working a small cave was found, of the limestone-fissure type. The mouth of this cave was about eight feet high and four feet wide, and the depth about four feet. It appeared that the patient had sheltered in the cave for his mid-day meal and had had a short sleep there afterwards. The cave showed evidence of habitation both by rats (nuts, seeds and olive-stones carefully gnawed through in the middle, with the kernel extracted) and by lizards (half-digested insect debris). There were various crevices in the walls, all of them shallow, except for one which appeared to be a rat-hole and which extended backwards for at least three feet. This hole was probably also used by lizards, for plenty of insect debris was collected from just inside the mouth; but from deeper down were recovered a gnawed melon-seed and a pellet which appeared to be vomited carob material. (Carobs, or locust-beans, are known to be eaten extensively by rats in Cyprus.) A spoon strapped to a stick was inserted into the hole, and samples of earth from various levels were removed and shaken through a 20-mesh wire sieve. Nine specimens of *Ornithodoros* at various stages of development were obtained. No ticks were found by sieving dusty earth from the floor of the cave, but in a shallow basin-like crevice in the wall Major Clark found a further specimen and some empty skins.

The other patient (Wood and Dixon's case 10) had been working a little further away. Several other small caves were examined, but no more ticks were found.

III. *Erimi*. Three of our cases occurred on this site (near Limassol), which was visited by both of us on October 19th, 1942. The camp was 200 yards south of the Limassol-Paphos main road, just east of Erimi bridge. A cook-house tent had been erected against a crumbling wall of an overgrown ruin, at a point where the wall contained a hollow about three feet above ground-level, some three feet high, four feet wide and two feet deep. At the back of the hollow was a hole with an array of faecal material full of insect debris (probably from a large lizard) arranged fan-wise four or five inches from the opening. Six inches inside the hole were found faecal pellets more likely to be those of a rat, together with a gnawed seed. It is probable that a lizard had at one time used the hole, and that its faeces had later been cleared out by the perhaps rightful occupant, a rat. Samples of the earth, which was of a dry and dusty nature, mixed with small stones, were examined with the same technique of spoon and sieve as described above. One *Ornithodoros* was found in this hole, and four more from inside and at the mouth of a similar but smaller hole in the same hollow.

IV (a). *Episkopi*. This is a village lying about half a mile west of Erimi. Eleven of our cases occurred among Indian troops of the same unit as that at Erimi, the men sleeping in tents on the sides of a low knoll just east of Episkopi village. One tent-site examined was a small depression in the lee of a large rock. A single specimen of *Ornithodoros* was found in earth from a crevice under the rock.

IV (b). *Episkopi*. Another site examined was a depression in the ground walled in by large rocks on the eastern edge of the village. One of our cases, an Indian soldier, slept there. Two ticks were obtained from a flat but deep crevice at the base of one of the rocks, which may have been a rat-hole, though no evidence of the presence of rats was found.

Both these sites were searched by us on October 19th, 1942.

V. *Ktima (Paphos)*. Four of our cases occurred among Indian troops living in huts in a gully approached by a track turning south from the Limassol-Paphos main road, just before the entrance to the town. This site was visited by both of us on October 20th, 1942. The track ran along the base of a cliff containing a number of holes, and at the far end, just opposite the huts, was a roomy cave used by some local inhabitant as a stable. Poultry were also kept in the cave. Two argasid larvae, together with some empty nymphal skins, were discovered by sieving debris, consisting of feathers, straw and fowls' faeces, from the floor of the cave. The larvae and skins were assumed at the time to be *A. persicus*, but when examined more fully later both were recognized as *Ornithodoros*. One of the larvae was freshly engorged; it was squashed, and a smear of the contained blood was examined after staining. Red blood-corpuscles of the non-nucleated mammalian type, 7μ in diameter, were clearly visible; no spirochaetes were seen.

VI. *Nicosta*. In November, 1943, a civilian Greek refugee boy was diagnosed, by the finding of spirochaetes in a blood film, as a case of relapsing fever. This boy, aged about 10 years, was living on the south-eastern outskirts of the town, in a house some 100 yards from the 57th General Hospital. One of us (R.M.G.) accompanied the Health Officer, Dr. Theodoulou, and the Health Inspector, Veyisi Effendi, when they went to investigate the case. Nothing incriminating was found either in the house or in the adjacent poultry-yard, but it was ascertained that the boy used to play in a nearby sheepfold—a depression in the ground walled in at one end and bounded on the remaining sides by a crumbling cliff containing a few caves. Here numerous *Ornithodoros* were found, not only in the caves, but in the earth along the base of the cliff. As there was not likely to be a rodent population sufficient to support so many ticks, it was assumed that they were also feeding on the sheep. In view of this host-difference, therefore, specimens were forwarded to Professor Adler, in case they should prove to be a different species. He reported that they were *O. tholozani*, identical with the previous specimens sent to him from Cyprus. It is of interest here to note that an Indian soldier, while an ambulant patient in the 57th General Hospital in December, 1942, contracted relapsing fever after 10 days in the hospital. It seems likely that he caught the infection in or near the sheepfold.

VII. *Sotira* (six miles south of Famagusta). During excavation of an old church in 1945, Mr. Mogabgab found some ticks which were identified by one of us (R.M.G.) as *O. tholozani*. Many of the men engaged on the work were bitten, usually on the feet, and most of those bitten became ill with fever. Blood films were not examined, and the men were treated for malaria, but from his fairly extensive experience of relapsing fever Mr. Mogabgab feels certain they were in reality suffering from that condition. Several became jaundiced during their illness, which was otherwise untreated and often protracted. Our informant states that the 'insects' found in the Famagusta excavations were exactly like the ticks found at this site.

B. SITES WHERE SEARCH FOR TICKS WAS UNSUCCESSFUL

I. *Famagusta*. ('Dungeon B.') Three cases of Wood and Dixon (1945) and one of ours occurred among troops billeted at different times in the old fortifications. Pre-war

clinical cases occurring in civilian employees of the Antiquities Department working in these sites have already been referred to. The site was visited by one of us (N.F.C.) at an early stage in the investigations, before the technique used in the later searches had been adopted. The site was rather difficult to search, as crevices between the stones in the walls of the old building were very numerous and mostly very narrow. There was no opportunity for repeating the search later, and military considerations prevented a proper investigation of other dungeons.

II (a). *Larnaca*. ('Cave C.') One case of Wood and Dixon (1945) was infected in a dug-out south of the town. This was visited by Wood, Dixon and one of us (R.M.G.) many months after the case occurred, and the dug-out had by then been partially dismantled.

II (b). *Larnaca*. Positions were visited by both of us south of the salt lake where one of our cases and one of Wood and Dixon's had occurred. The exact site of infection of the cases could not be ascertained.

III. *Yeroskipos* (near Paphos). Barracks where one of our cases had occurred were visited by both of us on October 20th, 1942. It was an unlikely site in which to find ticks, being a row of low huts on level ground with relatively recent dug-outs nearby and no old buildings. Swarms of fleas were the only insects found in the dug-outs.

IV. *Paphos*. A large earth-floored barn was visited by both of us near the harbour. It was used as living- and sleeping-quarters for Indian soldiers, among whom one of our cases occurred, and was a difficult place to search. There were several obvious but narrow and tortuous rat-holes at the bases of the internal rough stone walls, and a rat was actually seen in the barn.

C. SITES WHERE NO SEARCH WAS MADE

Many other cases of the disease occurred in widely separated localities throughout the island, which it was impossible for the authors to visit, or, in many cases, even to locate with any degree of exactitude. The following is a list of such sites arranged according to district.

Nicosia. 'Four miles west of Nicosia'; Lakatamia; Akaki; near Lefka; Kalo-khorio.

Kyrenia. 'Between Kyrenia and Myrtou'; Kyrenia Pass; Kormakiti.

Famagusta. Quarry north of the old fortifications, Famagusta (cf. civilian cases); Kondea area ('Cave F'); Vatali; Angastina; Lefkoniko Pass area; Milia (civilian case).

Limassol. The edge of Limassol aerodrome; Kalavassos (civilian cases).

Paphos. Paphos (tomb and one unknown site); hills between Paphos and Platres.

Larnaca. Dekelia.

D. SITES WHERE NO CASES HAD OCCURRED BUT WHERE SEARCH WAS MADE FOR TICKS

In several localities where, from a knowledge of the tick's habits, likely sites were found but where no cases of relapsing fever had occurred, search failed to bring to light any *O. tholozani*.

TRANSMISSION- AND CARRIER-EXPERIMENTS

Professor Adler's successful transmission-experiments with one of our ticks have already been discussed. This tick came from Lefka (site A, II). The present authors' attempts to transmit spirochaetosis to guinea-pigs by means of ticks were hampered by

a variety of factors, mostly concerned with the military situation. Either because of this, or for other reasons, all our experiments were unsuccessful, and in no case was a guinea-pig infected by the bite of a tick or by the injection of crushed ticks. Some of the difficulties met with in transmission-experiments have already been discussed. Chorine and Colas-Belcour (1944) found that ticks, although of a species known to carry relapsing fever spirochaetes, are sometimes immune to them. Only 3-4 per cent. of 'wild' ticks were found by Slavina (1944) to be naturally infected.

All the ticks used by us were half grown or adults. One female, after a lapse of three months in captivity, during which she was mated, laid eggs which hatched, but at that particular time, for reasons beyond our control, nothing could be done with the larvae, which unfortunately all died unfed. No other eggs were laid. The ticks were kept at room-temperature in glass tubes sealed with plugs of cotton wool. This was later learnt to be an unsatisfactory manner in which to keep them.

GUINEA-PIGS

Transmission-experiments with guinea-pigs will be briefly reported. The animals were in extremely short supply throughout the period of these studies; otherwise more would have been used. Those which died in February and March, 1943, did so of acute enteritis, epidemic at that time among the laboratory animals.

G.-P. I. Bitten by three ticks from Erimi (site A, III) on February 3rd and 4th, 1943. Two thick and two thin blood films were examined on February 8th; 12 thick blood films (from ear) were examined on February 12th, 16th and 18th. The animal was killed *in extremis* on the 18th; six brain smears were examined. No spirochaetes were found.

G.-P. II. Bitten by three ticks from Lefka (site A, II) on February 6th and 7th, 1943. Twelve thick blood films (from ear) were examined on February 12th, 16th, 20th, 23rd and 28th and on March 4th. Killed *in extremis* on March 6th. No spirochaetes were found.

G.-P. IV. Bitten by two ticks from Episkopi (site A, IV) on March 5th, 1943. Twelve thick blood films were examined on the 11th and 15th. Died on the 15th. Smears of coxal fluid were obtained on March 5th and examined. No spirochaetes were found.

G.-P. VIII. Bitten on October 14th, 1944, by one adult tick found near the 57th General Hospital (site A, VI). Died of unknown cause on October 28th. One thick blood film examined on the 21st and 24th. No spirochaetes were seen.

G.-P. X. Bitten on January 13th, 1945, by two ticks (male and female adults) found on site A, VI. One thick blood film examined on January 17th, 19th, 21st, 22nd, 24th and 25th. No spirochaetes were seen.

G.-P. XI. Bitten on February 2nd, 1945, by one adult male tick from site A, VI. One thick blood film examined on February 6th, 7th, 8th, 9th, 11th, 12th, 13th, 14th, 15th, 16th, 17th, 19th, 20th, 21st, 22nd, 24th, 25th and 26th. No spirochaetes were seen.

G.-P. XII. Bitten on February 27th, 1945, by two adult male ticks from site A, VI. One thick blood film examined on March 3rd, 4th, 6th, 7th, 8th, 9th, 10th, 12th, 13th and 14th. No spirochaetes were seen.

G.-P. XIII. Bitten on March 14th, 1945, by one male and one female adult tick from site A, VI. One thick blood film examined on March 19th, 21st, 22nd, 23rd, 24th, 26th, 27th, 28th, 29th and 30th. No spirochaetes were seen.

G.-P. VII. Inoculated subcutaneously on August 13th, 1942, with 0.5 c.cm. of saline emulsion of two ticks from Lefka (site A, II). Subsequent blood smears were all negative.

G.-P. IX. Inoculated subcutaneously on August 13th, 1942, with 1 c.cm. of saline emulsion of two ticks from Erimi (site A, III). Subsequent blood smears were all negative.

These negative results are not particularly surprising in view of the small number of ticks which could be used and the fact that they were all late-stage nymphs or adults. Had it been possible to work with the larvae when they hatched, results might have been different.

RATS

In addition to the spiny-mouse trapped at Kokkinitrimithia, 10 rats (*Rattus rattus*, or 'black rat') trapped in the old fortifications of Famagusta were forwarded alive by Captain Campion, R.A.M.C. Twelve thick blood films from the heart or external jugular vein were examined from eight of these rats and six thick blood films from the other two. No spirochaetes were seen. In a further effort to isolate a spirochaete, emulsions of the brains of nine of the rats were injected into guinea-pigs, as follows.

G.-P. III. Injected intraperitoneally on February 12th, 1943, with 1 c.cm. of brain emulsion from rat II, and on the 18th with 1 c.cm. of mixed brain emulsions from rats III, IV and V. Twelve thick blood films from the guinea-pig were examined on February 16th, 20th, 23rd and 28th and on March 2nd. Killed *in extremis* on March 2nd. No spirochaetes were seen.

G.-P. V. Injected intraperitoneally on March 17th, 1943, with 1 c.cm. of mixed brain emulsions from rats VI and VII, and on the 19th with 1 c.cm. of mixed brain emulsions from rats VIII and IX. Died on March 21st.

G.-P. VI. Injected subcutaneously on March 22nd, 1943, with 1 c.cm. of brain emulsion from rat X. Twelve thick blood films were examined on the 24th. Died on the 24th. No spirochaetes were seen.

MAN

One of us (R.M.G.) was accidentally bitten on November 5th, 1943, by larvae while investigating site A, VI. Eight larvae of *O. tholozani*, five of which were engorged, were later found in the clothing. Nine bites were counted on legs and ankles. These were mainly in front, probably owing to the kneeling position taken up while collecting the ticks. Only one of the bites was felt, as a slight prick, and in this instance the larva was observed *in situ* on the ankle. The bites were marked by circular flat spots, consisting of a dark-red central petechia of diameter slightly less than 1 mm., surrounded by a clearly demarcated paler-red area 2–3 mm. across. This outer zone varied a little in size in the different bites, and in some was entirely lacking. After four days some of the spots had completely disappeared, and the rest were much paler, although their details were still perfectly visible. None of the bites itched at any time. These observations tally fairly well with those of Adler *et al.* (1937) in the case of *O. tholozani*, but the bites of some ticks may immediately give rise to itching, as described by Lawrence and Terrell (1942) in the case of *O. turicata*.

Following this, the temperature was taken twice daily until November 20th; the highest recorded was 98.7° F. on the 15th, 10 days after the bites. On the 24th (nine days later) there was a moderately severe bilateral temporo-parietal headache running up to the vertex, which lasted for some hours, but without fever. Apart from this headache the patient felt perfectly well until December 1st (26 days after the bite), when the headache returned, more severe and now accompanied by fever (temperature 100° F.). Repeated blood films on December 1st and 2nd were negative (temperature up to 101.8° F.). He was admitted to Nicosia civil hospital on the 3rd, under the care of Dr. A. L. Fawdry, to whom we are indebted for some of the medical notes. There was now considerable headache, with much pain behind and on moving the eyes, and severe vomiting which lasted for 48 hours. There was mild photophobia and considerable malaise, but no shivering, only slight occasional nausea, and no other symptoms of note. This illness was unlike any which the patient had had before, including influenza, sandfly-fever, infective hepatitis and benign tertian malaria (which he had had in 1936 without subsequent relapse). No physical signs of disease were found, except a trace of albumen in the urine during the fever. Blood films were again negative on December 3rd, but were not afterwards repeated. Total blood leucocytes on December 3rd, 6,800; polymorphs 74 per cent., lymphocytes 15 per cent., basophils 3 per cent., mononuclears 8 per cent.

The fever and all symptoms were subsiding by December 5th. The bowels were not open from the 2nd until an enema on the 6th. The patient received no specific treatment and made a rapid recovery, being sent home symptom-free on December 8th. The fever lasted six days. The sites of the bites underwent no change during the febrile attack.

This may have been a mild attack of relapsing fever, although in the absence of a positive blood film no certain diagnosis can be made. The incubation-period would be rather a long one, although not outside the limit of some reported cases. It is possible, however, that, in this instance, the slight rise of temperature 10 days after the bites and the headache on the 19th day were abortive or subclinical attacks of spirochaetosis. Other possible diagnoses were sandfly-fever, influenza, glandular fever (the monocytes were not characteristic), malaria, infective hepatitis *sine* jaundice, and 'P.U.O.' i.e., any one of the short-term non-specific fevers seen in the Mediterranean area. Various arguments can be adduced against all these. Points in favour of relapsing fever were: (i) the patient was bitten by larval *Ornithodoros* ticks from a place where a proved case had recently occurred; (ii) no change in the polymorphonuclear count; (iii) relative monocytosis; (iv) rapid recovery from the paroxysm, without after-effects.

RESERVOIRS OF INFECTION

The purpose in examining wild rats was to find a reservoir of infection, though it is possible that there need be none. Davis (1943) has produced suggestive evidence that the tick itself may be a more efficient spirochaetal reservoir than the rodent host. Wheeler (1938) has noted that in nature ticks sometimes suck blood from other ticks. However, it seems likely, as pointed out by Darling (1922) and Moursund (1942), that in practice rodents often do act as reservoirs of infection. This does not rule out the possibility that the ticks are also reservoirs in themselves.

The findings of Catanei (1923) suggest that even man may sometimes be a carrier. Nicolle and Anderson (1927) postulate small mammals and ticks in a dual rôle as reservoirs.

Hindle (1935) discusses the literature on the subject of naturally infected small mammals. So far as they go, our observations tend to support the hypothesis that *O. tholozani* feeds mostly on small mammals, such as rodents, and that man is mostly an accidental host. Our observations cannot, however, exclude lizards and sheep as sometimes playing a similar rôle to the rodents. We have been unable to produce any evidence that any particular animals were acting as reservoirs of the infection.

THE HABITAT OF *O. THOLOZANI* IN CYPRUS

O. tholozani has been found in nine different sites in Cyprus, six of which were caves or hollows, two crevices under rocks, and one a ruined church. In all cases the sites were relatively dry, and they were often sandy. In three of the caves (including a hollow in a ruined wall) the ticks were obtained from holes which were assumed to be rat-holes, and in two of these there was evidence of occupation by both rats and lizards at different times. A cave where the tick was found on the roof showed rodent-tracks on the ground. Where ticks were found in a cave used as a stable and for poultry, examination of a smear of blood from a newly engorged larva showed that in this instance neither poultry nor lizards were the source. In one of our cases (caves in a sheepfold) it has been suggested that the ticks may also have fed on sheep.

From our observations it seems likely that *O. tholozani* behaves in Cyprus much as it does in Palestine, as reported by Adler *et al.* (1937). In Russia, on the other hand, it has also been found in cracks in the mud walls of animal-shelters and human dwellings, as recorded by Pavlovskii *et al.* (1929), and by Pavlovskii and Aluimov (1939).

In connection with Mr. Mogabgab's observation that relapsing fever and biting 'insects' (probably ticks) may be found in association with bat-guano or sheep-dung, it is interesting to note similar findings with regard to *Ornithodoros* in other parts of the world. Matheson (1935) described three new species (blood-suckers) taken from bats in Panama. Philip (1940) found two species, one of which was further described by Cooley and Kohls (1940), apparently living in guano in bat-infested caves in Arizona. Mazzotti (1941) found the same two species living under similar conditions in a cave harbouring bats in Mexico. (In neither case did these ticks show any evidence of blood-feeding.) Matheson (1941) found a new species in bat-caves in Venezuela. None of these ticks, however, has yet been shown to have any relation in nature with relapsing fever spirochaetes (Moursund, 1942). Other evidence that ticks may thrive in excreta is produced by Jellison (1940), who found engorged *O. parkeri*, a proved carrier of relapsing fever spirochaetes (Davis *et al.*, 1941), in the walls of nests of the burrowing-owl (*Speotyto cunicularia*), which lines its burrows with horse-manure often to a depth of 2-3 inches, in which material the ticks were found.

The fact that ticks have been found in association with bat-guano in underground sites in Cyprus does not, however, in any way exclude the possibility of rodents being the main hosts. Rats are known to be plentiful in the galleries of the Famagusta fortifications. The significance, if any, of the occasional proximity in nature of *Ornithodoros* to animal excreta is not known.

LONGEVITY OF TICKS

Nine half-grown nymphs and adults were kept alive in small glass test-tubes corked or tightly plugged with cotton wool from November 5th, 1943, the date of their capture, until October 15th, 1944. During this period of almost a year, they were

kept at room-temperature in Cyprus and on a journey to England and back, had no access to moisture, and were unfed. After October 15th, 1944, they were given a moist atmosphere. Three were dead by January 13th, 1945, and one more by February 2nd. Five were still alive on March 14th, after being fed at different times after October 15th, 1944. Of them, one male and one female lived unfed from November 5th, 1943, to January 13th, 1945 (435 days), one male unfed from November 5th, 1943, to February 2nd, 1945 (455 days) and two males unfed from November 5th, 1943, to February 27th, 1945 (480 days).

There are a number of records of ticks surviving for long periods of time, sometimes under adverse conditions. Manson and Thornton (1919) sealed some *O. moubata* in a test-tube and found them alive after nine months; Francis (1942) has kept *O. turicata* alive for five years starved and for nearly 10 years fed; and Pavlovskii and Skruinnik (1945) have shown that *O. tholozani* can resist starvation for up to 7½ years and can live for upwards of 25 years.

SUMMARY AND CONCLUSIONS

1. The history of relapsing fever in Cyprus is discussed. The first case to be diagnosed by the finding of spirochaetes in a blood film was in 1939. There is evidence, however, that the disease has been endemic for many years.

2. Cases began to occur among military personnel in August, 1941, and up to April, 1943, there were 98 such cases. Of these, all but seven had spirochaetes demonstrated in the blood. At least six proved civilian cases were recorded between 1939 and 1943. Many other very probable cases among civilians are known to have occurred between 1935 and 1945. The disease arose at points scattered over the island.

3. Clinically and epidemiologically the disease appeared to be of the tick-borne type. *Ornithodoros tholozani* (Laboulbène and Mégnin) was found in 1942 by Wood and Dixon in sites where men had contracted the fever. Thirteen localities where proved cases of the disease were believed to have been infected were examined by one or both of the present authors. *O. tholozani* was collected from eight of them, mostly in widely separated parts of the island. Altogether 29 cases (including six reported by Wood and Dixon and one civilian) occurred in sites where ticks were found. *O. tholozani* was also found at one site where a number of unproved civilian cases had occurred.

4. The incidence of the disease was mainly in units encamped in rocky places or on manoeuvres. Descriptions are given of the habitat of the ticks found. The most usual places in Cyprus for harbouring these creatures appear to be in or near dry shallow caves containing what are probably rat-holes. There is some evidence that *O. tholozani* may flourish in an environment containing animal excreta. It is possible that rats or similar rodents form the chief host on which the ticks normally feed. On one occasion they were found in a site where they may have been feeding on sheep.

5. One of our 'wild' adult female ticks, sent to Professor Adler, laid eggs which hatched to larvae which were found on first feeding to be capable of infecting guinea-pigs with relapsing fever spirochaetes by biting. This constitutes proof that *O. tholozani* from Cyprus can transmit relapsing fever and is sometimes naturally infected. Further proof that the disease in Cyprus may be tick-borne is provided by Dixon (1943), who has shown that the blood of a patient with relapsing fever was infective for guinea-pigs.

6. Transmission-experiments attempted by us, necessarily perfunctory, were all

negative. One of the authors developed a febrile illness, which may possibly have been relapsing fever, 26 days after receiving nine bites from larvae from a site where a proved case had recently occurred.

7. No evidence can be offered as to the reservoir of infection in Cyprus.

8. Some of our ticks were kept alive unfed—for much of the time under very adverse conditions—for upwards of 480 days.

9. The first cases of relapsing fever in Cyprus correctly diagnosed were in civilians, but had it not been for the recent war, and its consequent influx of troops encamped in rural areas or on manoeuvres over the countryside, the wide-spread potential incidence of the disease in the island might not have been so dramatically exposed.

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FIELD TRIALS WITH NEW ANTIMALARIAL DRUGS IN EGYPT

BY

A. HALAWANI,

I. BAZ

AND

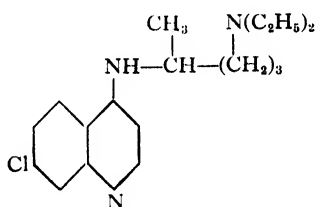
F. MORCOS

(From the Fouad I Institute of Research and Hospital for Tropical Diseases, Cairo)

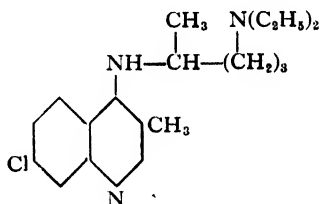
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INTRODUCTION

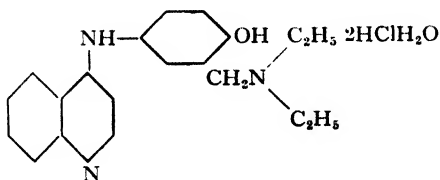
The antimalarials known under the names of chloroquine (resochin or aralen), nivaquine C (santochin) and camoquine (CAM-AQI) are 4-aminoquinoline derivatives. Their formulae are shown below, and it may be seen that the basic side-chain in the 4-position of the first two compounds is identical with that in the 8-position of pamaquin. In camoquine a hydroxyaniline nucleus is incorporated in the basic side-chain of the 4-position. Nivaquine C differs from chloroquine only by the inclusion of a methyl group in the 3-position. Paludrine, a biguanide, has, of course, a fundamentally different chemical constitution from that of the other three compounds (Curd, Davey and Rose, 1945).



I Chloroquine



II Nivaquine C



III Camoquine

PRESENT INVESTIGATIONS

Patients were treated, according to the schedules described below, under ambulatory conditions in field stations. Cases which gave histories of repeated attacks, with no tendency for spontaneous remission, were chosen for treatment. Drug administration was carried out by the present authors, and the patients returned to their homes either by bus or by walking a mile or two. Thick and thin blood smears were examined daily during treatment and afterwards once a week for approximately six months. The great

majority of our cases were not only suffering from malaria but were also heavily infested with ancylostome, ascaris and bilharzia worms. The high splenic index among them could not therefore be ascribed to malaria alone, as intestinal bilharziasis is one of the chief causes of splenomegaly in Egypt. The great majority (78 per cent.) were suffering from marked anaemia. During the time of treatment and since, intensive control-measures against larvae and adults of the vector (*Anopheles pharoensis*) have been carried out in the whole area of Khanka, where our patients resided. Nevertheless, the possibility of reinfection could not be entirely excluded. To ascertain the true relapse-rate of malaria after administration of drugs in a country like Egypt would necessitate keeping patients in a hospital away from infective mosquitoes for a period of a year, which, for obvious reasons, would be very difficult to do. Our various groups of patients received treatment during the same period (July-September, 1947) and resided under the same social and environmental conditions.

RESULTS

I. CAMOQUINE

Camoquine was tested by us in 1946 on 42 cases (Halawani, Baz and Morcos, 1947*a*). Of these, 39 patients were suffering from benign tertian and three from malignant tertian malaria. Four different schedules of treatment were tried out, with apparently satisfactory results. Two cases received the whole course in 12 hours, i.e., four tablets (0.05 gm. each) in the morning and four in the evening, with no deleterious effects. Most of the cases, however, received an initial dose of two tablets, followed by another two every 12 hours for three doses, i.e., a total of eight tablets totalling 0.4 gm. of camoquine. Of the 30 cases treated and followed up, 29 were benign tertian and one was malignant tertian. They were all apparently cured, and all parasites disappeared from their blood after, at most, five days. Weekly examinations of these patients and of their blood, over a period varying in individual cases between three and five months, revealed no clinical or parasitological relapses. Five of these cases, however, who were followed up for the rest of the year, were found to be suffering from overt malaria and benign tertian parasitaemia 215-300 days after treatment.

During 1947 we treated another series of patients with the same drug according to a new schedule devised by the senior author. Five tablets of 0.05 gm. each were administered to each patient morning and evening for one day only, i.e., a total of 0.5 gm. of the drug. Ninety cases received this short course of treatment, the ages of the patients varying from 15 to 53 years and their weights from 34 to 75 kgm. Splenic enlargement was present in 40 per cent. of the cases, but this was not of purely malarial origin, because, as mentioned above, bilharziasis was common. Of the 90 cases, three were suffering from malignant tertian malaria. One of these cases exhibited rings only in the peripheral blood; on the day following treatment all rings disappeared. In the other two cases also the rings disappeared on the day following treatment, but the crescents persisted in one case for four days and in the other for nine days. The remaining 87 cases were all benign tertian malaria exhibiting rings and gametocytes in the blood smears. Of these, 85 were followed up. Fever and asexual stages of the parasites disappeared on the day following treatment; gametocytes disappeared in 51 cases on the second day, in 30 cases on the third day, and in four cases on the fourth day following treatment.

Toxic symptoms were more marked in this group of cases than in the series treated the previous year with the same drug with lower dosage or with a course extending over a longer period. In a few cases headache was severe and persisted for two days, but left no ill effect. Nausea, vomiting, abdominal colic and diarrhoea were encountered in some cases. A few patients complained of pain in the loins, but no pathological findings were detected in the urine. Fainting and giddiness were also complained of by some of the patients. On the whole, however, the toxic symptoms were not serious and disappeared completely after cessation of treatment. These cases were followed up for a period of six months, during which time four exhibited overt malaria associated with benign tertian parasitaemia.

II. CHLOROQUINE

Chloroquine was investigated on ambulatory patients at the Khanka Malaria Research Station. To each of 42 patients an initial dose of four tablets, i.e., 1 gm. of the drug, was administered at mid-day, followed by two tablets at 8 p.m., two tablets at noon on the following day, and two more 24 hours later, i.e., a whole course of 10 tablets, totalling 2.5 gm. of the drug, was given within 48 hours. Of this series, six cases were malignant tertian and the remaining 36 benign tertian. The blood of all these cases except two (one benign tertian and one malignant tertian) was examined by the thick-smear method once weekly for six months. All cases exhibiting malignant tertian ring-forms only in their peripheral blood, and all benign tertian cases exhibiting sexual and asexual stages, became free from parasites and symptoms in 1-3 days after the commencement of treatment. Four cases in the group were found to be suffering from overt malaria with benign tertian parasitaemia 204, 247, 285 and 308 days after treatment. One of these cases relapsed three times after freedom from malaria for 204 days; he was given mepacrine on April 23rd, 1946, and exhibited benign tertian parasites on June 7th of the same year; a course of nivaquine was administered, but fever and benign tertian parasitaemia reappeared two months later.

Toxic symptoms due to chloroquine treatment were trivial, of rare occurrence, and of brief duration. They consisted of vomiting and headache.

Showing the percentage of clinically cured cases of benign tertian

Examination	Nivaquine C (93 cases)				
	Trophozoites	Gametocytes	Trophozoites + gametocytes	Negative	Percentage
Before treatment	2	14	77	0	0
After 1st day's treatment ...	0	43	4	46	49
" 2nd " " " ...	0	12	0	81	95
" 3rd " " " ...	0	0	0	93	100
" 4th " " " ...	0	0	0	93	100
" 5th " " " ...	0	0	0	93	100
" 6th-10th days' treatment	0	0	0	93	100
Schedule of dosage	18 tablets in 5 days given as follows : 1st day : 5 tablets 2nd " : 4 " 3rd-5th days : 3 " (Total 1.8 gm.)				
Signs of toxicity	None				

III. NIVAQUINE C

The antimalarial activity of nivaquine C was investigated on 96 ambulatory cases of malaria. The following schedule of treatment was adopted :

First day : 3 tablets (0.1 gm. each) in the morning ; 2 in the evening.

Second day : 2 " " " " " ; 2 " "

Third to fifth day : 2 " " " " " ; 1 " "

Of the 96 cases thus treated, 95 were benign tertian and one was malignant tertian. This latter case harboured rings and crescents ; after the first day of treatment the rings only disappeared from the peripheral blood, but after the third day of treatment the patient did not appear for further examination. Of the 95 benign tertian cases, 93 were followed up for a period of 2-5 months after treatment, and were regularly examined clinically and parasitologically at weekly intervals. Pyrexia and parasites disappeared after the first day of treatment in 46 cases, and after two days in the remainder ; gametocytes took a few days longer to disappear. The drug was well tolerated by all the patients, and no toxic symptoms were observed after its administration. Nine cases of the whole group re-exhibited malaria with benign tertian parasites after periods varying from 32 to 70 days. One other case was found to be suffering from benign tertian parasitaemia 238 days after treatment.

IV. PALUDRINE

Paludrine was administered as one tablet (0.1 gm.) three times daily for 10 days. One hundred and twenty cases received this course of treatment, of which six were malignant tertian malaria and 114 were benign tertian. Of the malignant tertian cases, three exhibited rings only in the peripheral blood ; all parasites disappeared after the course, but two and a half months later rings and crescents reappeared in the blood of one of them ; the other cases were not followed up, but it was observed that crescents did not disappear during the course of treatment. Of the 114 benign tertian cases, 98 were followed up for a period of six months, and one of them relapsed during that period.

No toxic symptoms or ill effects were observed following paludrine treatment.

the administration of nivaquine C, camoquine and paludrine

Camoquine (85 cases)					Paludrine (98 cases)				
pho- ites	Gameto- cytes	Trophozoites + gametocytes	Negative	Per- centage	Tropho- zoites	Gameto- cytes	Trophozoites + gametocytes	Negative	Per- centage
0	1	84	0	0	0	25	74	0	0
0	34	0	51	60	1	64	14	20	20
0	4	0	81	96	0	49	0	50	50
0	0	0	85	100	0	5	1	93	84
0	0	0	85	100	0	3	0	96	97
0	0	0	85	100	0	1	0	98	99
0	0	0	85	100	0	0	0	99	100
0 tablets in 2 doses : 5 in the morning, 5 12 hours later (Total 0.5 gm.)					1 tablet t.d.s. for 10 days, i.e., a whole course of 30 tablets (Total 3 gm.)				
headache, giddiness, nausea, vomiting, diarrhoea, abdominal pains (all mild symptoms)					None				

TABLE II

Showing the number of cases who suffered from overt vivax malaria or parasitaemia after treatment with nivaquine C, camoquine and paludrine. Weekly examinations of patients extended over a period of nine months after treatment

Compound	No. of cases treated	Schedule of treatment	No. of cases which re-exhibited overt malaria or parasitaemia after treatment during an observation-period of		Average no. of days which elapsed between treatment and reappearance of malaria during an observation-period of	
			Six months	Nine months	Six months	Nine months
Nivaquine C	93	1.8 gm. in 5 days	9	13	54	113
Camoquine	85	0.5 gm. in 1 day	4	10	49	156
Paludrine	98	3 gm. in 10 days	1	16	70	237

DISCUSSION

In February, 1947, we published our preliminary results on the chemotherapeutic activity of camoquine (synonym CAM-AQI) on a series of 42 cases of malaria. In the present report, observations on another series of 85 cases are recorded. In our first trials, various schedules of treatment were used, but to the second group of patients we administered the drug in two doses of 0.25 gm. each, allowing an interval of 12 hours between the first and second dose. In October, 1947, Mein and Rosado reported to the Brazilian Congress of Hygiene on their experience of new medicaments against malaria in the 'Amazone' programme. They stated that when they first began to use camoquine there was no accepted dosage, and that initially they administered it in a single dose of 10 tablets, or 0.5 gm., to adults, and of five tablets, or 0.25 gm., to children. Later they reduced the dose to 0.4 gm. for adults and 0.1-0.2 gm. for children. Adopting the sterilization of the peripheral blood as their criterion in assessing the efficiency of the drugs tested, they found that, in cases of malaria caused by *Plasmodium falciparum*, camoquine sterilized the blood in 72 hours (15 cases), paludrine in 96 hours (five cases), and oxichloroquine in 72 hours (nine cases), and that chloroquine failed to sterilize the blood in five out of six cases. In cases of malaria caused by *P. vivax* they found all the drugs efficient in clearing all stages of the parasite from the peripheral blood—camoquine in 30 hours after the beginning of treatment (39 cases), chloroquine in 48 hours (14 cases), oxichloroquine in 24-72 hours (six cases), and paludrine in 48-72 hours (two cases). In our second series of tests against vivax malaria all stages of the parasite disappeared from the peripheral blood in 72 hours after the administration of camoquine (85 cases) and in 144 hours after paludrine (98 cases) (see Table I). Three of our malignant tertian cases responded to camoquine on the second day; pyrexia ceased and rings completely disappeared, but crescents continued to appear in thick films for four days after treatment in one case and for nine days in another.

Of chloroquine Loeb *et al.* (1946) have reported satisfactory results after doses of 0.6 gm. followed by 0.3 gm. 6-8 hours later and by 0.3 gm. on the following day, as well as by the administration of 1.2 gm. divided over a period of 24 hours. For suppressive

therapy a dose of 0.3 gm. given on the same day each week is recommended. Sapero (1946) states that chloroquine is three times as active as quinacrine hydrochloride and that it prolongs the intervals between relapses beyond that observed with either quinine hydrochloride or quinacrine. Most *et al.* (1946) report that, during a total period of 120 days, relapse-rates were in the case of quinine 90 per cent., in that of quinacrine hydrochloride 82 per cent., and in that of chloroquine 75 per cent.; they recommend a total dose of 1.5 gm. of chloroquine administered over a period of four days. As mentioned above, the present authors tested chloroquine (2.5 gm.) on the Egyptian strain of vivax malaria. Weekly examinations of our patients and of their blood smears during a period of 3-6 months revealed no clinical or parasitological relapse, though four out of 36 benign tertian cases re-exhibited parasitaemia associated with typical tertian fever 204, 247, 285 and 308 days after treatment. Although we believe that these were true relapses, we prefer to refer to them as the malaria incidence-rate, since reinfection among treated cases cannot be excluded. Loeb *et al.* (1946) state that chloroquine does not prevent relapses in vivax malaria even when administered in doses many times greater than those required to terminate an acute attack; nor will it prevent the establishment of vivax infection when administered as a prophylactic. Generally speaking, our own experience with the drug so far is that, in the case of the Egyptian strains of *P. vivax*, relapses are fewer than are recorded for other strains.

Nivaquine C was administered to adult patients by Durand, Decourt and Schneider (1944) in daily doses of 0.3 gm. for five days. The cases were followed up for a year without a single relapse being observed, but the numbers treated were insufficient for any definite conclusions to be drawn. In our own series of 93 cases of vivax malaria nivaquine C was found to be a potent drug against erythrocytic stages, its action being mainly schizonticidal. Nine cases in the series re-exhibited parasitaemia after an average period of 54 days.

Paludrine has been subjected to clinical trials at the Liverpool School of Tropical Medicine (Adams *et al.*, 1945; Maegraith *et al.*, 1945) and in Australia (Fairley *et al.*, 1946). According to Covell (1947), it is yielding promising results in India, and in our hands in Egypt it has also given satisfactory results. According to Johnstone (1946) paludrine, given in a 10-day course, is effective in treating acute attacks of benign tertian malaria, but is in no way comparable with the standard quinine-pamaquin treatment in controlling further relapses. There is a suggestion, however, that paludrine given in a 10-day course causes longer periods of freedom from relapse than does quinine-pamaquin, in spite of the high relapse-rate. Andrews *et al.* (1947) found that the relapse-rate after the administration of paludrine agreed closely with that obtained by Johnstone. In our series of 98 vivax cases one re-exhibited parasitaemia after 70 days. Of two falciparum cases which were followed up, however, one re-exhibited malignant tertian rings 77 days after treatment. According to Andrews *et al.* (1947), the average time of relapse after a course of 500 mgm. paludrine b.d. for 14 days was 60 days, the number of proved relapses being 10 and the number of clinical relapses three out of 35 cases.

Although we do not wish to commit ourselves to any conclusions regarding the relapse-rate in our series, we have found that the incidence-rate of parasitaemia (during six months) among cases treated with paludrine was less than in the series treated with camoquine and with nivaquine C. Camoquine, however, administered in one or two doses, sterilizes the blood in a comparatively short time—an advantage which renders the drug suitable for field-work.

TOXICITY

The symptoms of acute toxicity due to camoquine in animals are vomiting, clonic-convulsions, and respiratory failure causing death; the symptoms of chronic intoxication are anorexia, loss of weight, vomiting or diarrhoea (or both). In our series, toxic symptoms in man were rare and of brief duration; they included vomiting, diarrhoea, tenesmus, headache, palpitations and dizziness. The symptoms observed following doses of chloroquine include mild and transient headache, visual disturbance, pruritis, vomiting, and gastro-intestinal complaints. In our series of cases these symptoms were trivial, of rare occurrence and of brief duration. As regards nivaquine C, following the average therapeutic doses used by us not a single case of intolerance was observed. Paludrine also was well tolerated by our patients and we encountered none of the toxic symptoms recorded in the literature as occurring after large doses (1.0 gm. daily)—symptoms such as the presence of red blood-cells, sheets of epithelial cells or hyaline casts in the urine, vomiting, or transient increase in myelocytes.

The incidence of malarial parasitaemia among the groups treated by the various drugs during an observation-period extending over six months is recorded in Table II.

SUMMARY

1. With the object of finding the most suitable drug for administration on a large scale in antimalarial field-work, camoquine, chloroquine, nivaquine C and paludrine were tested in Egyptian vivax and falciparum malaria.

2. Over 400 patients were treated with the compounds. All four drugs proved efficacious in clearing the blood of parasites of benign tertian and malignant tertian malaria. Disappearance of parasites occurred earlier after the administration of chloroquine, nivaquine C and camoquine than after paludrine.

3. No serious toxic effects were observed during six months following the administration of the drugs. Paludrine and nivaquine C were extremely well tolerated. Mild transient headache and vomiting occurred occasionally after the administration of chloroquine. Camoquine on rare occasions caused vomiting, diarrhoea, tenesmus, headache, palpitation and dizziness. These symptoms were of brief duration and of no consequence. In view of the fact that the drugs were administered to groups of patients suffering from bilharziasis, ancylostomiasis, ascariasis and anaemia in addition to malaria, we consider that they are safe in the therapeutic doses recommended.

4. The lowest incidence of parasitaemia, in an observation-period of six months, occurred among the group treated with paludrine. Camoquine has the advantage of being administrable in a single day.

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RECORDS OF FILARIA INFECTIONS IN MOSQUITOES IN CEYLON

BY

HENRY F. CARTER

(*Medical Entomologist, Ceylon*)

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The data recorded in this paper have accrued over several years from work done in various parts of Ceylon and the Maldive Islands. Much of the work was undertaken, in association with investigations instituted by the Department of Medical and Sanitary Services of Ceylon, in areas where human filariasis was severely endemic. Information on some of these investigations has already been published by Carter (1933), Sweet and Dirckze (1934), and Dassanayake (1938, 1939); and in a recent article Carter (*in the press*) discusses the evidence available concerning the transmission of rural filariasis (*Wuchereria malayi*) and the infections found in mosquitoes of the genus *Taeniorhynchus* (*Mansonioides*). The latter findings are also included here, in order that the records now given shall be complete to the time of writing.

These records all relate to filaria infections found in mosquitoes caught under natural conditions; no experimental infections in laboratory-bred mosquitoes are included. So far as possible the results obtained from different localities have been grouped in relation to the known endemicity of the human parasites, *W. malayi* and *W. bancrofti*, but it does not by any means follow that all the infections recorded from endemic areas are necessarily of human origin. In all probability many were derived from domestic animals, but our knowledge of the differential characters of larval filariae in mosquitoes is still imperfect and in many cases does not allow of accurate identification. Nevertheless, there is reason to believe that in some localities at least—notably in Toppur and Colombo—a high proportion of the parasites found in the mosquitoes were of human origin.

The investigations to which these records refer were carried out at various times in the North-Western Province (12 rural areas), the Eastern Province (the village of Toppur), the Western Province (Colombo suburbs) and the most southerly of the atolls comprising the Maldive Islands. The results are summarized in Table I, and brief notes are given on the work done in each district.

NORTH-WESTERN PROVINCE

The investigations undertaken in the North-Western Province, in so far as they relate to the mosquitoes of the subgenus *Mansonioides*, have been noticed elsewhere (Carter, *in the press*). They include three series of observations: one series made from April, 1937, to March, 1938, at nine widely separated villages (Munneswaram, near Chilaw; Hettipola; Wariyapola; Hiripitiya; Nikaweratiya; Kuliypitiya; Malla-wapitiya, near Kurunegala; Ridigama; and Wegama); a second series made at two villages (Bandarakoswatte and Magulagama) where filariasis was severely endemic, over a period of 3½ years from May, 1937, to November, 1940; and a third series at the non-endemic village of Akaragama from May, 1932, to March, 1933.

In the first two investigations mosquitoes were collected from the villages at least once each month, over a period of three or four days, by hand-catching in the houses in the mornings, and by trapping (using human and animal baits) in the evenings from 6.30 p.m. to 8.30 p.m. In the investigations at Akaragama mosquitoes were collected during the night from cattle-baited traps only.

In a filarial survey of the province carried out by the Department of Medical and Sanitary Services in 1937, nearly 70 endemic foci of the disease were located. Over 4,000 blood films were examined, and all parasites found were identified as *W. malayi*; the mean microfilaria-rate for the infected villages was 27.5. Of the villages named above, four were registered as endemic foci of the disease—Munneswaram (microfilaria-rate 5.6), Hiripitiya (rate 4.0), Bandarakoswatte (rate 47.5) and Magulagama (rate 44.9)—and two (Hettipola and Wariyapola), which had not been examined, were situated in known endemic areas where neighbouring villages showed microfilaria-rates of from 16.7 to 56.8. In Table I the findings in the mosquitoes captured from these six villages have been combined (columns 2-4). No precise data relating to the remaining six villages named are available, but the evidence indicates that few clinical cases were present in any of the areas in which they are situated. None was registered as an endemic focus of the disease.

In the first series of observations (April, 1937, to March, 1938) several thousand mosquitoes were captured and 8,500 were dissected and examined. Of these, 4,640 were from the four villages (Munneswaram, Hettipola, Wariyapola and Hiripitiya) situated in endemic areas, and 3,860 were from the remaining five villages in this series. *T. (M.) uniformis* was much more prevalent in the first group of villages than in the second, forming approximately 40 per cent. of the total catch in the first case and only 6 per cent. in the second. Filaria infections were found in 31 mosquitoes from the endemic villages and in 11 from the others. These infections occurred in *T. (M.) uniformis* (20), *T. (M.) indianus* (1), *Aedes pseudomediofasciatus* (9), *Ae. lineatopennis* (6), *Ae. pallidostriatus* (4) and *Ae. pipersalatus* (2); 34 of the infected mosquitoes were captured in cattle-baited traps, seven in human-baited traps, and one in a house. Proboscis infections were observed in all species except the single infected specimen of *T. (M.) indianus*. Of the infected *T. (M.) uniformis*, 17 were obtained from the endemic villages and three from the villages in non-endemic areas; 13 of these infected mosquitoes were collected during April-June, 1937. Almost all (92 per cent.) of the specimens of *Ae. lineatopennis* collected in this investigation were obtained from the village of Munneswaram, the majority in October, when six (infection-rate 2.9) were found infected with filaria larvae.

In the second series of observations, made at Bandarakoswatte and Magulagama, experimental control of *Mansonioides* mosquitoes by systematic removal of the plant *Pistia stratiotes* from all tanks and pools in the vicinity of Bandarakoswatte was introduced in May, 1937. During the observation-period over 35,000 mosquitoes were collected, the numbers obtained from the two villages being approximately the same. *T. (M.) uniformis* was less prevalent in the experimental village during the years 1938 and 1939, but increased again in 1940. Approximately 32 per cent. of the total catch for both villages was obtained from houses, 10 per cent. from human-baited traps, and 58 per cent. from cattle-baited traps. The predominant mosquitoes were *Anopheles subpictus*, *Culex tritaeniorhynchus*, *Ae. (A.) pallidostriatus*, *T. (M.) uniformis* and *Ae. (A.) pipersalatus*, which together formed nearly 80 per cent. of the total catch. In the houses *A. subpictus* was far more prevalent

than any other species, constituting almost 90 per cent. of the mosquitoes caught therein; *A. culicifacies*, *C. fatigans* and *C. (Lophoceratomyia) minutissimus* were also found with some frequency, but other species, including *T. (M.) uniformis*, were rare. The catch from the human-baited traps consisted mainly of *A. subpictus* (63 per cent.), *T. (M.) uniformis* (14 per cent.) and *C. tritaeniorhynchus* (5 per cent.). In the cattle-baited traps many different species of mosquitoes were captured, the most prevalent being *C. tritaeniorhynchus*, *Ae. (A.) pallidostriatus*, *T. (M.) uniformis* and *Ae. (A.) pipersalatus*, in the order named; but *A. subpictus*, *A. hyrcanus*, *Ae. (A.) pseudomediofasciatus*, *C. fuscocephalus*, *C. gelidus* and *C. whitmorei* were not uncommon. Infections with filaria larvae were found in 83

Filarial inf

Species of mosquito	North-Western Province, rural					
	From endemic foci (6) of <i>W. malayi</i>			From villages (5) w ^h endemicty of <i>W. m</i> was slight		
	Dissec- tions	Infec- tions	Rate	Dissec- tions	Infec- tions	
<i>Culex fatigans</i>	657	—	—	394	—	
" <i>gelidus</i>	877	—	—	378	—	
" <i>tritaeniorhynchus</i>	3,733	4	0·16	808	—	
" <i>bitaeniorhynchus</i>	100	1	1·0	13	—	
" <i>fuscocephalus</i>	848	1	0·12	288	—	
" <i>sitiens</i>	73	1	(1·4)	—	—	
<i>Armigeres obturbans</i>	127	3	2·4	25	—	
<i>Taeniorhynchus (Mansonioides) uniformis</i>	4,361	60	1·4	219	3	
" (M.) <i>uniformis</i> and <i>T. (M.) indianus</i> *						
" (M.) <i>indianus</i>	65	2	(3·1)	12	1	
" (M.) <i>annuliferus</i>	286	—	—	11	—	
<i>Aedes (Aedimorphus) pipersalatus</i>	1,590	16	1·0	43	—	
" " <i>pallidostriatus</i>	2,676	5	0·19	719	—	
" (<i>Banksinella</i>) <i>lineatopennis</i>	846	7	0·8	4	—	
" (<i>Aedes</i>) <i>pseudomediofasciatus</i>	1,342	3	0·2	775	7	
<i>Anopheles hyrcanus</i> var. <i>nigerrimus</i>	421	8	1·9	—	—	
" <i>barbirostris</i>	42	1	(2·4)	—	—	
" <i>subpictus</i>	2,192	2	0·1	—	—	

* Not differentiated.

Rates based on less than 100 mosquitoes dissected are given in brackets.

Other mosquitoes examined for filaria in these investigations but found negative were: *C. whitmorei* (744), *C. mimulus* (48), *C. (L.) minutissimus* (182), *C. (L.) fuscanus* (29), *Ae. (Mucidus) scatophagoides* (147), *Ae. (S.) a*

mosquitoes—32 (in 8,325 dissected) from Bandarakeswate and 51 (in 8,932 dissected) from Magulagama. The sites of capture of these infected mosquitoes were: houses 4 (infection-rate 0·2 per cent.), human-baited traps 19 (1·2 per cent.), cattle-baited traps 60 (0·44 per cent.). The mosquitoes in which infections occurred were: *T. (M.) uniformis* (43), *Ae. (A.) pipersalatus* (14), *A. hyrcanus* (8), *C. tritaeniorhynchus* (4), *Armigeres obturbans* (3), *T. (M.) indianus* (2), *A. subpictus* (2), and *C. sitiens*, *C. bitaeniorhynchus*, *C. fuscocephalus*, *Ae. (A.) pseudomediofasciatus*, *Ae. (A.) pallidostriatus*, *Ae. (B.) lineatopennis* and *A. barbirostris* (1 each). Of the infected *T. (M.) uniformis*, 18 were obtained from Bandarakeswate (734 mosquitoes dissected; rate 2·5) and 25 from Magulagama

(1,747 dissected; rate 1.4); 31 of the infected females were caught during the months April-July inclusive. Proboscis infections were found in five mosquitoes—*T. (M.) uniformis* (1, in March), *Ae. (A.) pipersalatus* (2, in July and December), *A. hyrcanus* (1, in June), and *Armigeres obturbans* (1, in October).

From May, 1932, to March, 1933, the village of Akaragama (near Kurunegala) was used as an entomological field station for studies in connection with malaria. The work on filaria was of an incidental nature only, and, although there is reason to believe that the great majority of the infections found in the mosquitoes were not of human origin, it is considered that the observations made are of sufficient interest to be worthy of record.

			Eastern Province, rural area			Western Province, Colombo suburbs			Maldivic Islands, rural area		
From non-endemic area (Akaragama)			Endemic focus of <i>W. malayi</i>			Endemic focus of <i>W. bancrofti</i>			Endemic focus of <i>W. bancrofti</i>		
-	Infections	Rate	Dissections	Infections	Rate	Dissections	Infections	Rate	Dissections	Infections	Rate
253	—	—	—	—	—	995	87	8.7	159	11	6.9
91	—	—	18	1	(5.5)	298	—	—	—	—	—
94	—	—	67	—	—	267	—	—	7	—	—
			1	—	—	3	—	—			
			9	—	—						
170	7	4.1				1	—	—	11	1	(9.1)
30	1	(3.3)				87	1	(1.1)	15	—	—
						148	2	1.3			
			445	20	4.5						
			50	6	(12.0)						
			577	27	4.7	6	—	—			
						3	—	—			
						11	—	—			
18	2	(11.1)									
036	160	15.5	14	—	—	3	—	—			
12	2	(16.6)				1	—	—			
27	—	—	132	—	—	19	—	—			

albopictus (29), *A. culicifacies* (290), *A. vagus* (137), *A. varuna* (212), *A. tessellatus* (216), *A. annularis* (286), *A. pal-*
32) and *A. jamei* (142).

In addition to the investigations indicated in the table, 545 mosquitoes—including 263 *C. fatigans*, 88 *T. (M.) uni-*
 61 *T. (M.) annuliferus*—were examined from villages in other parts of Ceylon; one female of *T. (M.) uniformis*
 mature filaria larvae in the head.

The mosquitoes were caught at fixed hours during the night, from 7 p.m. to 6 a.m., in a trap baited with a buffalo calf; the trap was used on an average of 10 nights each month during the observation-period. Over 7,000 mosquitoes were captured, 24 species of culicines and 12 species of anophelines being represented. Of the culicines, *C. fatigans*, *C. whitmorei*, *C. gelidus*, *C. tritaeniorhynchus* and *Armigeres obturbans* were the most prevalent; of the anophelines *A. hyrcanus* var. *nigerrimus* greatly predominated (forming approximately 75 per cent. of the total anopheline catch), but *A. varuna* and *A. tessellatus* were not uncommon. Approximately 2,500 mosquitoes were dissected and examined, and 172 were found to harbour filaria larvae (see Table I). In the three infections found

in *Ae. (A.) pseudomediofasciatus* and in *T. (M.) uniformis* young filaria larvae in the thoracic muscles only were present, but in the others mature larvae were seen and all appeared to be of similar structure. These mature larvae were relatively large—from 2.2 to 2.5 mm. in length—and were devoid of the characteristic caudal protuberances of the human and canine species. It is possible that they were derived from the village buffaloes which were used extensively for agricultural purposes. Infections in *A. hyrcanus* were found continuously throughout the observation-period, even during months when the prevalence of this mosquito was very low. They increased greatly in September and October (infection-rates 36.8 and 14.3), coincidental with a sharp rise in mosquito prevalence, but thereafter were less numerous, although from December to February *A. hyrcanus* was still present in considerable numbers. Proboscis infections were found in 15 females of this mosquito—in September (2), October (9), December (2) and February (2). The records of infections found in *A. hyrcanus* throughout the observation-period are given in Table II.

TABLE II
Filaria sp. incert. in *A. hyrcanus* var. *nigerrimus*,
Akaragama, North-Western Province

Month	Average catch of <i>A. hyrcanus</i> per night	No. of <i>A. hyrcanus</i> dissected	No. of <i>A. hyrcanus</i> with filaria
May (1932)	0.3	2	1
June	3.0	9	2
July	2.1	16	9
August	0.5	3	2
September	39.5	228	84
October	26.2	245	35
November	6.4	30	1
December	21.9	208	13
January (1933)	26.1	105	4
February	22.1	177	8
March	1.4	13	1

EASTERN PROVINCE (TOPPUR)

Toppur is a Muslim village (population about 1,500) situated at the northern extremity of a large lake (Allai Tank) about 25 miles south-east of Trincomalee by road. Filariasis due to *W. malayi* is severely endemic, and conditions in this respect have shown little change for many years, Bahr reporting a microfilaria-rate of 26.6 in 1914, and Carter rates of 34.4 in adults and 25.0 in children in 1933, with elephantiasis in 29 (22.7 per cent.) of 128 adults examined at random.

In October and November, 1932, when investigations were undertaken, mosquitoes were abundant, and of a total of 1,944 collected over 70 per cent. were species of *Taeniorhynchus* (*Mansonioides*). Seventeen other species of culicine mosquitoes and four species of anophelines were also represented in the catch, but none was present in large numbers. *C. fatigans* was not found.

Over 1,300 mosquitoes were dissected and examined for filaria; the results of these dissections are given in Table I. *Filaria* larvae were found in 54 mosquitoes, all but one of which were species of *Taeniorhynchus*. Proboscis infections were present in six of these

mosquitoes—*T. (M.) annuliferus* (1), *T. (M.) indianus* (2), and *T. (M.) uniformis* and *T. (M.) indianus* (not differentiated) (3). The remaining infection with filaria occurred in *C. gelidus*, a single mature larva being found in the abdomen; this larva, however, differed in structure from those seen in *Taeniorhynchus*, which appeared to be *W. malayi*.

WESTERN PROVINCE (COLOMBO SUBURBS)

Although cases of filariasis have been reported from time to time over many years from Colombo and its environs, the data available did not, until recently, suggest that the incidence of the disease was sufficiently great to be of serious moment. Of late, however, reports from various sources have indicated that the incidence of the disease is increasing, more particularly in the suburban areas, which of recent years have been the scene of considerable building activity, resulting in a large accession of the smaller types of bungalow. Towards the end of 1947, therefore, the Department of Medical and Sanitary Services arranged for a detailed enquiry into the disease to be undertaken in the suburban areas. A comprehensive report on the subject will, no doubt, be published later, but for the purpose of these records it need only be stated that at the time of writing over 2,000 blood films have been examined and 130 have shown microfilariae. All the microfilariae found were identified as *W. bancrofti*. In the southern suburbs the mean microfilaria-rate was 8.9 (range 6.7–11.9), in the eastern suburbs 3.1 (range 0–8.5), and in the northern suburbs 11.9 (range 10.0–16.2).

The entomological part of the investigation was carried out from January to April, 1948, and consisted essentially of the collection, identification and examination for filaria larvae of mosquitoes from houses in representative areas in most of the suburbs, and the examination of potential breeding-places of mosquitoes in the vicinity of each site.* Night-trapping of mosquitoes, with cattle as bait, was also undertaken at some of the sites. The approximate positions of the sites where the field-work was done are shown in the accompanying map, and the main results of the examinations are given in Tables I and III.

From Table III it will be seen that in the houses *C. fatigans* was the dominant mosquito, constituting in most localities 90 per cent. or more of the total catch. It was particularly prevalent in houses at Nugegoda, Dehiwala, Mount Lavinia, Kalubowila, Kirillapone and Welikada, where the hand-catching rates for the species ranged from approximately 20 to 40 per man-hour. Infections with filaria larvae were found in 87 mosquitoes, all of which were *C. fatigans*; the mean infection-rate was 8.8. Infected mosquitoes were found in all the suburbs visited except Narahenpita, which is not truly urban in character and where, at the time when the work was done, mosquitoes were scanty and few *C. fatigans* females were caught. Elsewhere, the mosquito infection-rates varied from 2.2 to 21.0, the highest rates occurring at Mattakkuliya (where, however, the number of *C. fatigans* examined was small), Dehiwala, Mount Lavinia and Kolonnawa.

The mosquito catches from the cattle-baited traps were in marked contrast to those from the houses at the same sites. *C. fatigans* was in great minority or was absent from the collections made. The predominant mosquitoes were *C. tritaeniorhynchus*, *C. gelidus*, *T. (M.) uniformis* (mainly from Kirillapone) and *Armigeres obturbans*. Only three mosquitoes—two *T. (M.) uniformis* and one *Ar. obturbans*—in over 700 dissected contained filaria larvae.

* The mosquito field-work was very ably carried out by Mr. S. R. da Silva, Field Assistant in the Division of Medical Entomology.

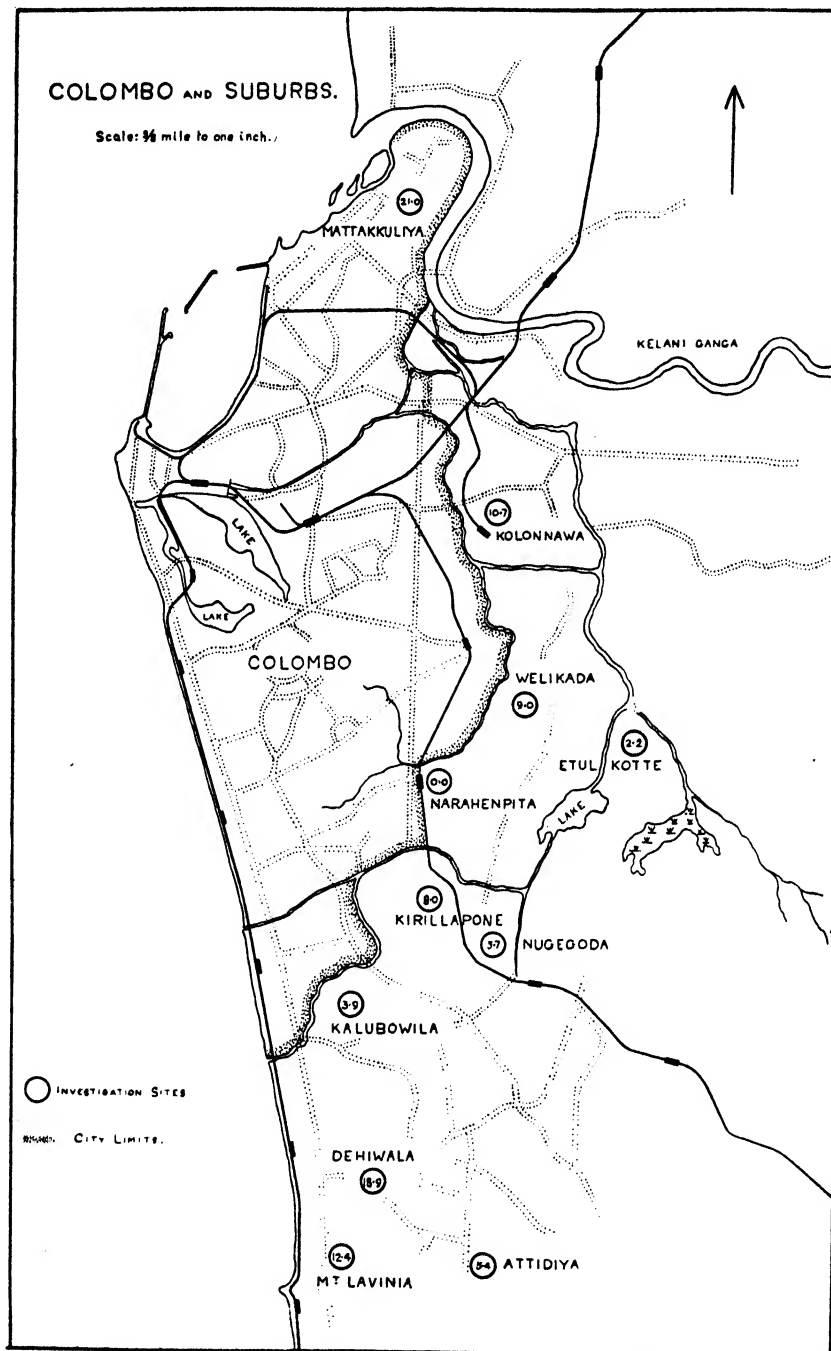
Of the 87 infected females of *C. fatigans* obtained from the houses, 14 harboured mature filaria larvae, eight mature larvae and developing forms in the thoracic muscles, and 65 developing forms only. Mature larvae were present in the proboscis of seven females (0.7 per cent. of those dissected). The number of mature larvae present was usually from one to five, but exceeded 10 in four mosquitoes; the largest number seen in any one female was 30—proboscis 3, head 4, thorax 7, abdomen 16. Of the mosquitoes harbouring developing forms only, approximately 60 per cent. showed less than 10, and 17 per cent. 20 or over; the largest number counted was 68. The mature larvae were from 1.3 mm. to 1.8 mm. in length, and all showed the three caudal protuberances which are regarded as characteristic of *W. bancrofti*. The developing forms ranged from 0.2 mm. to 1.1 mm. in length and from 0.015 mm. to 0.05 mm. in width.

TABLE III
Filariar infections in mosquitoes, Colombo suburbs

Suburb	Houses								Cattle-baited traps				
	Houses examined	Mosquitoes caught		No. of <i>C. fatigans</i> caught	Mosquitoes dissected	<i>C. fatigans</i> dissected	Infections (all <i>C. fatigans</i>)	Infection-rate (<i>C. fatigans</i>)	Mosquitoes caught		No. of <i>C. fatigans</i> caught	Mosquitoes dissected	Infections
		No.	No. per hour						No.	No. per hour			
<i>South</i>													
Kalubowila	50	260	27.7	246(♂106)	132	127	5	3.9					
Dehiwala	31	207	37.6	199(♂85)	117	111	21	18.9					
Mount	27	149	31.0	140(♂41)	96	89	11	12.4					
Lavinia													
Kirillapone	135	604	23.9	540(♂181)	344	301	24	8.0	266	66.5	4(♂1)	174	} <i>Ar. obturbans</i> (1), <i>T. (M.) uniformis</i> (1) Nil Nil
Nugegoda	30	241	42.3	240(♂98)	123	122	7	5.7	64	32.0	1	55	
Attidiya	31	79	13.2	65(♂24)	45	37	2	5.4	83	41.5	Nil	81	
<i>East</i>													
Narahenpita	66	117	9.9	35(♂18)	64	17	Nil	Nil					
Welikada	30	140	24.6	115(♂39)	88	67	6	9.0					
Etul Kotte	28	108	21.2	101(♂55)	45	45	1	2.2	212	106.0	Nil	125	} <i>T. (M.) uniformis</i> (1) Nil
Kolonnawa	31	84	16.1	75(♂18)	64	56	6	10.7	232	116.0	Nil	185	
<i>North</i>													
Mattakkuliya	27	95	18.2	39(♂20)	48	19	4	21.0	229	114.5	Nil	106	Nil
Totals	486	2,084	—	1,795(♂685)	1,166	991	87	8.8	1,086	—	(5♂1)	726	3

Of the three infected mosquitoes caught in the cattle-baited traps, two—*T. (M.) uniformis*—harboured only small developing forms in the thoracic muscles, and these could not be identified; but the third—*Ar. obturbans*—showed three mature larvae in the proboscis. These larvae were from 2.16 mm. to 2.35 mm. in length and 0.03 mm. in width, with the caudal extremity rounded and devoid of protuberances; presumably they were not human parasites.

These investigations showed clearly that filariasis due to *W. bancrofti* was wide-spread throughout the suburbs of Colombo, and that the essential carrier of the disease was the common house mosquito *C. fatigans*. This mosquito was breeding prolifically in polluted



MAP of Colombo and vicinity showing approximate positions of investigation centres. Figures within the circles denote the filaria infection-rate found in *C. fatigans*.

water in catch-pits, built and unbuilt drains, pits containing refuse, and old tins, chatties and other discarded receptacles (see Plate XI). The prevalence of the disease and of the mosquito carrier is indeed a direct result of defective sanitation, due largely to lack of a water-carriage system for sewage disposal.

MALDIVE ISLANDS

The records given for the Maldivé Islands relate to the most southerly of the atolls situated just south of the equator; they were obtained during a brief visit made in the latter part of February and early March, 1943. Although no records of the incidence of filariasis on this atoll existed, some indication of its prevalence and of the aversion in which it was held by the residents was gained from the fact that for long the local practice had been to segregate persons with elephantiasis to a particular islet within the group. As a result of this concentration of infected individuals and their families, there was ample ocular evidence of the prevalence of the disease on the islet reserved for segregation, but comparatively little in villages on the neighbouring islands; in fact, in the latter the presence of the disease was largely masked.

In a random sample of 84 residents (14 adults and 70 children) of the village of Hittadu, one adult only had elephantiasis (left leg and scrotum), but three others showed microfilariæ (*W. bancrofti*) in the blood. In a similar sample of 23 adults and 25 children and youths living in segregation, all the adults and nine of the children had elephantiasis and two showed microfilariæ. Unfortunately, circumstances prevented the blood films from being taken late at night; had this been possible higher microfilariæ-rates would probably have been obtained.

Mosquitoes were not abundant at the time of the visit, and *C. fatigans* was the only species commonly found in the houses. No species of *Taeniorhynchus* (*Mansonioides*) were found, and lack of suitable breeding-places renders their presence on the atoll improbable. Collections of mosquitoes were made from the houses in three villages on separate islands, and 196 females (159 of which were *C. fatigans*) were examined for filaria larvae. Twelve of these (11 *C. fatigans* and one *C. sitiens*) were infected; no proboscis infections were seen, but mature and active larvae were present in two female *C. fatigans* and in the infected *C. sitiens*. In *C. fatigans* collected from the village of Hittadu the infection-rate was 6.6, and in those from the segregation island 8.0; no infections were found in 33 mosquitoes collected from the remaining village, but this was not surprising, since the source of infection—the villagers—had been removed from the island several months previously. It may be noted here that scarcely any domestic animals and few wild ones—other than rats, birds and flying foxes—occur on this atoll.

SUMMARY

1. Records, collected over a period of several years, of filaria infections in mosquitoes caught in various parts of Ceylon and the Maldivé Islands are summarized.
2. The records are grouped in relation to the known endemicity of human filariasis (*Wuchereria malayi* and *W. bancrofti*) in the localities from which the mosquitoes were collected, but it is not suggested that all infections from endemic areas were of human origin.

BREEDING - PLACES OF *Culex fatigans*, THE CARRIER OF FILARIASIS, IN THE SUBURBS OF COLOMBO



FIG. 1. Catch-pit and drain, private latrine.



FIG. 2. Catch-pits, communal latrines.



FIG. 3. Cement drain in disrepair.



FIG. 4. Drain; water stagnant and heavily polluted.

3. From known endemic foci of *W. malayi* in the North-Western and Eastern Provinces of Ceylon, infections were found in 15 species of mosquitoes, but a large proportion (about two-thirds) occurred in species of the subgenus *Mansonioides*.

4. In localities in the North-Western Province where filariasis due to *W. malayi* was slight or absent, *Mansonioides* mosquitoes were less prevalent, but some were found infected. Many infections, however, occurred in other mosquitoes, notably in *Anopheles hyrcanus*, in which at one period (September, 1932) an infection-rate of 36·8 was observed. The infections in this mosquito were not due to *W. malayi* or *W. bancrofti* and were probably derived from the village buffaloes.

5. In localities where *W. bancrofti* was endemic—the suburbs of Colombo and villages in some of the Maldiv Islands—the great majority of the infections found occurred in *Culex fatigans*. Infected mosquitoes were obtained from houses in all but one of the suburban areas of Colombo examined, the infection-rates ranging from 2·2 to 21·0, with a mean rate of 8·8. All the mature filaria larvae found in *C. fatigans* presented the characteristics of *W. bancrofti*.

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TAXONOMY OF THE ETHIOPIAN SANDFLIES (*PHLEBOTOMUS*)

III.—NEW SPECIES AND RECORDS: ALTERATIONS AND ADDITIONS TO THE KEYS

BY

R. KIRK

AND

D. J. LEWIS

(From the Stack Memorial Research Laboratories, Khartoum)

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Since we prepared illustrated keys for the identification of the Ethiopian species of *Phlebotomus* (Kirk and Lewis, 1946) several new species and records have been reported from the Ethiopian region. The purpose of the present paper is to add the new records to the list of Ethiopian species previously given, and to indicate such alterations in the keys as are necessary for their inclusion therein. In addition, we have incorporated in the keys three species which were only briefly mentioned in previous papers, *P. hirtus*, *P. eremitis* and *P. dubius*. *P. hirtus* may ultimately prove to be a Palearctic rather than an Ethiopian species, but *P. eremitis* has now been identified in the Sudan. *P. dubius* may be a synonym of *P. antennatus* Newstead.

In our previous paper an error occurs in the key for the females of the subgenus *Prophlebotomus*, section 11 (page 123), in the words 'Fourth palpal segment longer than third.' This should read 'Fourth palpal segment shorter than third.' The error has been eliminated in the additions and alterations to the keys given below.

For convenience of reference the illustrations in this paper are numbered in a continuous series with those in our previous paper.

NEW SPECIES AND VARIETIES OF *PHLEBOTOMUS* FROM THE ETHIOPIAN REGION

P. (Phlebotomus) lesleyae Lewis and Kirk.

P. lesleyae, Lewis and Kirk (1946), *Proc. R. Ent. Soc. Lond.* (B), 15, 55 (♂ ♀).

This species is evidently a member of the subgenus *Phlebotomus*, with some unusual characters, especially the spermatheca, which is a thin-walled sac without annulations.

Distribution. Sudan.

P. (Sintonius) calcaratus Parrot.

P. calcaratus, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 137 (♂ ♀).

P. calcaratus very closely resembles the Indian species *P. christophersi* Sinton, from which it differs in the presence of spines similar to those of *P. wansoni* on the anterior and posterior femora of both sexes, and in other minor differences.

Distribution. Sudan, Eritrea.

P. (Prophlebotomus) hunti Lewis and Kirk.

P. hunti, Lewis and Kirk (1946), *Proc. R. Ent. Soc. Lond.* (B), 15, 55 (♂ ♀).

A distinctive species, with no large buccal teeth, although numerous punctiform teeth of the 'anterior' type and a well-marked pigmented area are present in both male and female.

Distribution. Sudan.

P. (Prophlebotomus) horgani Lewis and Kirk.

P. horgani, Lewis and Kirk (1946), *Proc. R. Ent. Soc. Lond.* (B), 15, 55 (♂ ♀).

This species resembles the Abyssinian *P. wurtzi*, from which, however, it shows some clear differences. The spermatheca is a thin-walled sac, without the fine parallel transverse ridges described in *P. wurtzi*. Both sexes have a pigmented area, and the buccal cavity of the female has at least five delicate pointed teeth, with no small punctiform teeth.

Distribution. Sudan.

P. (Prophlebotomus) cowlandi Lewis and Kirk.

P. cowlandi, Lewis and Kirk (1946), *Proc. R. Ent. Soc. Lond.* (B), 15, 55 (♂).

The female is unknown. The male runs to *P. congolensis* in our key to the males of the subgenus *Prophlebotomus*, but it is easily differentiated from all the known Ethiopian species by the buccal armature.

Distribution. Sudan.

P. (Prophlebotomus) ruttledgei Lewis and Kirk.

P. ruttledgei, Lewis and Kirk (1946), *Proc. R. Ent. Soc. Lond.* (B), 15, 55 (♀).

The male is unknown. The buccal armature and pigmented area of the female have some resemblance to those of *P. congolensis*, from which, however, it is easily separated by the pharynx, which resembles that of *P. caffrarius* De Meillon and Lavoipierre.

Distribution. Sudan.

P. (Prophlebotomus) hirtus Parrot and de Jolivière.

P. hirtus, Parrot and de Jolivière (1945), *Arch. Inst. Pasteur Algér.*, 23, 56 (♂).

The female is unknown. The male differs from all the known Ethiopian species in having seven spines on the distal segment of the superior clasper.

Distribution. Hoggar.

P. (Prophlebotomus) eremitis Parrot and de Jolivière.

P. eremitis, Parrot and de Jolivière (1945), *Arch. Inst. Pasteur Algér.*, 23, 56 (♂ ♀).

This species closely resembles *P. africanus* var. *niger*, from which it is distinguished by the palpal formula, which is 1, 2 (3, 4), 5 instead of 1, 2, 3, 4, 5. Other differences are the character of the pharynx in the female and the position of the non-deciduous seta on the distal segment of the superior clasper of the male.

Distribution. Hoggar, Sudan.

P. (Prophlebotomus) pastorianus Parrot, Mornet and Cadenat.

P. pastorianus, Parrot, Mornet and Cadenat (1945), *Arch. Inst. Pasteur Algér.*, 23, 281 (♀).

The male is unknown. The female resembles *P. signatipennis* and *P. occidentalis*, but

has a palpal formula of 1, 2, 3, 4, 5 instead of 1, 2, 4, 3, 5, and there are other minor differences.

Distribution. French West Africa (Guinea).

P. (Prophlebotomus) guiraudi Kervan.

P. guiraudi, Kervan (1946), *Ann. Parasit. Hum. Comp.*, 21, 155 (♀).

This species was described from one female only, which closely resembles *P. schwetzi*. From the description and figures we are unable to separate it from that species.

Distribution. French Sudan.

P. (Prophlebotomus) aretasi Kervan.

P. aretasi, Kervan (1946), *Ann. Parasit. Hum. Comp.*, 21, 155 (♂).

This species is known by one male only, which is distinguished by the buccal armature, which has 16–18 teeth with irregular contours and milled or serrated edges, as in the lateral teeth of *P. cowlandi* (fig. 65).

Distribution. French Sudan.

P. (Prophlebotomus) crosarai Parrot and Wanson.

P. crosarai, Parrot and Wanson (1946), *Rev. Zool. Bot. Afr.*, 39, 225 (♀).

The male is unknown. The spermatheca of the female resembles that of *P. africanus* but *P. crosarai* is readily distinguished from *P. africanus* and from other members of the subgenus *Prophlebotomus* by the unusual antennal formula.

Distribution. British Congo.

P. (Prophlebotomus) richardi Parrot and Wanson.

P. richardi, Parrot and Wanson (1946), *Rev. Zool. Bot. Afr.*, 39, 225 (♀).

The male is unknown. The female resembles *P. pastorianus*, from which it can be differentiated by its smaller size, the alar index, and the number of the buccal teeth.

Distribution. Belgian Congo.

P. (Prophlebotomus) kirki Parrot.

P. kirki, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 121 (♂ ♀).

Parrot (1948) points out the close resemblance between this species and the Indian *P. purii* Sinton, *P. zeylanicus* Annandale and *P. sylvestris* Sinton, from all of which it is, however, readily distinguished by the morphology of the buccal cavity and other characters. Among African species *P. kirki* resembles *P. ingrami* and *P. serratus*.

Distribution. Sudan.

P. (Prophlebotomus) lewisi Parrot.

P. lewisi, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 125 (♀).

The male is unknown. The female is closely related to *P. squamipleuris*, from which it differs clearly in the antennal formula, the absence of spines on the spermathecae, and the morphology of the buccal armature.

Distribution. Sudan.

P. (Prophlebotomus) affinis var. *vorax* Parrot.

P. affinis, Lewis and Kirk (1940), *Proc. R. Ent. Soc. Lond.* (B), 9, 127 (♂).

P. affinis var. *vorax*, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 142 (♂ ♀).

The male of this form was described by us (1940) under the name *P. affinis* Theodor. Parrot (1948) reported the presence of spines on the femora, a feature subsequently found also in the type. The female of var. *vorax* differs from the type in the larger number of buccal teeth and spermathecal segments. The male of *P. affinis* is still unknown.

Distribution. Sudan.

P. (Prophlebotomus) schoutedeni var. *pungens* Parrot.

P. schoutedeni var. *pungens*, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 145 (♀).

The female of this variety differs from the species *P. schoutedeni* in its greater size, in the uniformity of the buccal teeth, and in the wider and more heavily armed pharynx. The male is unknown.

Distribution. Sudan.

P. (Prophlebotomus) schwetzi var. *nigricans* Parrot.

P. schwetzi var. *nigricans*, Parrot (1948), *Arch. Inst. Pasteur Algér.*, 26, 269 (♀).

The female of this variety differs from the type in its greater size, in the greater length of the third segment of the antenna, in the deeper pigmentation of the buccal region, in the higher value of Aiii/E (1.1, as compared with 0.7–0.85), and in the antennal formula, which is 1, 2, 3, 4, 5 instead of 1, 2 (4, 3), 5. The male is unknown.

Distribution. Sudan.

ADDITIONAL RECORDS OF DISTRIBUTION

In addition to the new species and varieties given above, the distribution of some of the other Ethiopian species has been made more precise by the inclusion of more localities. The new records of distribution are given below.

P. (Phlebotomus) roubaudi: French Sudan, French Niger.

P. (Sintonius) clydei: Senegal, French Niger, French Sudan, Eritrea.

P. (Sintonius) subtilis: Sudan, Eritrea.

P. (Sintonius) wansoni: French Sudan.

Subgenus *Prophlebotomus*

P. africanus: Senegal, French Sudan, French Guinea, Middle Congo (French).

P. africanus var. *niger*: French Sudan, French Guinea, Dahomey, Ivory Coast.

P. africanus var. *sudanicus*: Senegal.

P. buxtoni: Sudan.

P. cinctus: Sudan.

P. collarti: Middle Congo (French), Sudan.

P. congolensis var. *distinctus*: Senegal, French Guinea, Dahomey.

P. dubius: Senegal, French Sudan, French Guinea, Ivory Coast.

P. dureni: Ivory Coast, Middle Congo (French), Sudan.

P. occidentalis: Sudan.

P. signatipennis: Senegal, French Sudan, Ivory Coast, Dahomey, Eritrea.

P. schoutedeni: Dahomey.

P. simillimus: Dahomey.

P. schwetzi: Senegal, French Guinea.

P. squamipleuris: Senegal, French Sudan, Ivory Coast, Dahomey, Middle Congo (French).

P. squamipleuris var. *inermis*: Sudan.

SYNONYMY

The form recorded by us as *P. langeroni* var. *orientalis* has been raised to specific rank as *P. orientalis* by Parrot and Clastrier (1946). Parrot, Mornet and Cadenat (1945) now conclude that the distinctive characters of *P. schwetzi* var. *aethiopicus* are not sufficiently constant to justify maintenance of varietal rank for that form. In the same paper these authors described as *P. raptus* a single female from French Guinea, but, after examining several similar specimens from the Sudan, Parrot (1948) considers that *P. raptus* is a synonym of *P. ingrami* Newstead. The descriptions in Parrot's later papers differ in some minor points from the description by Adler, Theodor and Parrot (1929) of the female of *P. ingrami*, which was based on a single specimen from Uganda; they should be regarded as complementing the earlier description. As mentioned in our previous paper (Kirk and Lewis, 1946), Parrot (1939) has drawn attention to the close similarity between *P. wansoni* Parrot and *P. matadiensis* Theodor, suggesting that they may be identical. Parrot (1948) now states that this is so, and *P. matadiensis* therefore becomes a synonym of *P. wansoni*. Parrot and Durand-Delacre (1947) have recorded that *P. viator* Parrot and Martin is a synonym of *P. clydei* Sinton. These alterations may be summarized thus:

<i>P. langeroni</i> var. <i>orientalis</i>	= <i>P. orientalis</i>
<i>P. schwetzi</i> var. <i>aethiopicus</i>	= <i>P. schwetzi</i>
<i>P. raptus</i>	= <i>P. ingrami</i>
<i>P. matadiensis</i>	= <i>P. wansoni</i>
<i>P. viator</i>	= <i>P. clydei</i>

Dr. Parrot, in a private communication, has drawn our attention to the close similarity between the males of *P. katangensis* Bequaert and Walravens and of *P. martini* Parrot, which differ only in the character of the hairs on the peduncle of the proximal segment of the superior clasper. These hairs are of uniform large size in *P. katangensis*, whereas in *P. martini* there are small hairs as well as large ones, though the small hairs are very inconspicuous and might easily be overlooked. It is possible that these two forms may be identical, in which case *P. martini* would become a synonym of *P. katangensis*; but the matter can only be decided after re-examination of *P. katangensis*, which at present is known by only one male.

TERMINOLOGY

The terminology used by most authors for the description of the male terminalia of *Phlebotomus* is that of Newstead (1911). Christophers and Barraud (1926) established the homology of the hypopygium of *Phlebotomus*, which in the male is rotated through an angle of 180° after emergence of the imago. Tonnoir (1935) and Theodor (1947) have pointed out that retention of the older nomenclature is inconsistent with the rules now generally accepted in entomological literature, and have suggested that the terminology should be altered to that indicated by Christophers and Barraud.

The present writers have consistently employed the terminology of Newstead; but

some authors now use that of Christophers and Barraud, which will probably ultimately supersede the older system. Equivalent terms in the two systems of nomenclature are therefore given below.

NEWSTEAD	CHRISTOPHERS AND BARRAUD
Proximal segment of superior clasper	= Coxite
Distal segment of superior clasper	= Style
Intermediate appendage	= Parameres
Intrromittent organ	= Penis (penis filaments + penis sheath)
Inferior claspers	= Lateral lobes
Submedian lamellae	= Cerci

Tonnoir (1935) has also suggested that the antennal geniculated spines of Newstead should be called 'ascoids,' on the grounds that Feuerborn has shown them to be sense-organs, comparable to those found in other Psychodidae. This suggestion has been adopted by Theodor (1947) in a recent paper.

ADDITIONS AND ALTERATIONS TO THE KEYS

The alterations required to include the new records in the keys for the identification of the Ethiopian species (Kirk and Lewis, 1946) are given below. They comprise substitutes for some of the existing sections, together with new sections to be incorporated in the keys. The substitutes are indicated by numerals, which refer to the sections in the original keys requiring alteration. The new sections are indicated by numerals combined with letters, in order to define clearly their position in the keys.

PHLEBOTOMUS FEMALES

1. Females with numerous erect hairs on dorsal aspect of abdominal segments II-VI, usually in tufts at the distal end of the segment.
Buccal armature and pigmented area absent or rudimentary ... 2
2. Spermatheca segmented in entire length ... 3
Spermatheca sac-like, not segmented ... 2a
- 2a. Alar index = 5 or 6. A specialized, very large, cave-dwelling species *P. gigas*
Alar index = 0.3 ... *P. lesleyae*

PHLEBOTOMUS MALES

9. Distal segment of superior clasper very elongated, having parallel sides, and bearing 5 short spines. Inferior clasper bearing 2-6 apical spines. Intermediate appendage with 3 characteristic lobes (fig. 14) ... 10
Distal segment of superior clasper irregular in shape, bearing 5 long spines ... 10a
10. Inferior clasper with 4-6 very short terminal spines (fig. 16) ... *P. roubaudi*
Inferior clasper with 2 long terminal spines (fig. 14) ... *P. papatasi*
- 10a. Intermediate appendage simple ... 11
Intermediate appendage consisting of an upper lobe bearing hairs, a lower lobe composed of a stem bearing a few hairs, and a thin broad smooth plate (fig. 59) ... *P. lesleyae*

SINTONIUS FEMALES

7. Pigmented area and buccal armature poorly developed, the latter with approximately 6 widely spaced teeth (fig. 73) ... *P. calcaratus*
 Buccal armature and pigmented area well-developed, with more than 10 buccal teeth ... 7a
- 7a. Buccal teeth less than 20 in number, sharply pointed, with points widely spaced. Resembles *P. clydei* (fig. 41), but without anterior teeth ... *P. meilloni*
 Buccal teeth 40 or more, straight, parallel, arranged in a palisade. Pigmented area extending across whole width of buccal cavity (as in fig. 42) ... 8
9. Buccal teeth 55-60. Pigmented area in the form of a segment of a circle, convex anteriorly, with a pointed anterior triangular process *P. thomsoni*
 Buccal teeth about 50. Pigmented area consists of a broad triangular part, with a blunt irregular anterior process and with a number of corrugated lines converging into the narrow anterior part ... *P. wansonii*

Delete section 10.

SINTONIUS MALES

4. Buccal armature a row of small teeth, separated into small groups of 3-6 teeth, the central ones being larger than the lateral ones. Anteriorly there is an irregular row of about 6 small punctiform teeth (fig. 35) ... *P. clydei*
 Buccal armature consisting of 13-14 teeth, the lateral ones being longer than the central ones, and having their points directed towards the mid line. Anterior teeth in two lateral groups of 6-8 teeth; between these a group of small punctiform teeth ... *P. subtilis*
 Buccal armature consisting of 5 widely separated teeth, the outer two being pointed, the median three broad and with serrated edge. Numerous anterior punctiform teeth arranged in groups of 3-4 teeth (fig. 72) ... *P. calcaratus*
6. Four spines on the distal segment of the superior clasper all terminal *P. affinis*
 Two of the four spines on the distal segment of the superior clasper markedly subterminal, the other two being terminal. Small non-deciduous seta arises distally near origin of terminal spines ... 7
7. Pigmented area broad, triangular, with apex directed anteriorly and base extending across whole width of buccal cavity. Buccal armature has about 35 parallel teeth ... *P. thomsoni*
 Pigmented area small, central, turnip-shaped. Buccal armature has 10 broad teeth with serrated edges ... *P. meilloni*

Delete section 8.

PROPHLEBOTOMUS FEMALES

5. Spermathecae rounded or turnip-shaped. Two well-developed lateral protuberances are present anteriorly to the buccal armature (fig. 43) ... 5a
 Spermathecae in the form of sacs. No lateral protuberances anteriorly to buccal armature ... 5b
- 5a. Spermathecae bearing transverse rows of small spines. Antennal formula 1/IV-XV ... *P. squamipleuris*
 Spermathecae smooth without spines (fig. 58). Antennal formula 2/III-XV ... *P. lewisii*

- 5b. Spermatheca sac-like, with many fine parallel ridges. Buccal cavity without pigmented area, provided with 3 pointed median teeth and on either side a number of small irregularly arranged punctiform teeth ... *P. wurtzi*
 Spermatheca a thin-walled sac. Buccal cavity with about 5 delicate pointed teeth; pigmented area narrow, with irregular posterior margin (fig. 63) ... *P. horgani*
 Spermatheca sac-like, constricted in the middle. Buccal teeth 12-14, arranged fanwise, the median teeth being larger than the lateral ones. A specialized cave-dwelling species ... *P. mirabilis*
6. Spermathecae more or less cylindrical capsules with wide ducts (fig. 7) ... 7
 Spermathecae oval or elliptical capsules with narrow ducts (fig. 8) ... 7b
7. Pharynx heavily armed, cordiform and pigmented (fig. 29). Antennal formula 1/IV, 2/V-XV ... *P. similimus*
 Pharynx not cordiform or heavily pigmented ... 7a
- 7a. 10-12 equal and pointed teeth in buccal cavity, with a row of anterior punctiform teeth, each of which is placed at the interval between two teeth. Pharynx three times as wide posteriorly as anteriorly ... *P. collarti*
 12-14 unequal buccal teeth, the median ones much narrower than the lateral ones, which are very wide (fig. 44). No anterior teeth. Pharynx slender ... *P. decipiens*
 22-24 pointed teeth in buccal cavity, the median ones bigger than the lateral ones and forming a well-marked salient projecting backwards (fig. 71) ... *P. kirki*
- 7b. No large teeth in buccal cavity, only small punctiform teeth, arranged in 2-3 rows with additional punctiform teeth at the sides ... *P. huntii*
 Large teeth present in buccal cavity ... 8
8. Antennal formula 2/III-XV ... 9
 Antennal formula 2/V-XIII, 1/XIV-XV. Buccal armature with 18-20 pointed equal teeth, and 1, 2, or sometimes 3 rows of anterior punctiform teeth ... *P. crosarai*
10. Ant. III = IV + V. Alar index > unity ... *P. freetownensis*
 Ant. III < IV + V. Alar index < unity ... 10a
- 10a. Palpal formula 1, 2 (3, 4), 5 ... *P. eremitis*
 Palpal formula 1, 2, 3, 4, 5 ... *P. africanus* and vars.
11. Pharynx cordiform. Palpal formula 1, 2, 4, 3, 5 ... 12
 Pharynx not cordiform. Palpal formula 1, 2, 3, 4, 5 ... 13
12. Buccal teeth 22-28 ... 12a
 Buccal teeth less than 20 or more than 30 ... 12b
- 12a. Buccal teeth 22-26. Pigmented area in the form of a straight band or ellipse, with an anterior helmet-shaped prolongation (fig. 47) ... *P. signatipennis*
 Buccal teeth 24-28, arranged in an arc concave posteriorly, the 6-8 median teeth being smaller than the 3 or 4 immediately lateral to them, which are also larger than the 2 or 3 extreme lateral teeth on each side. At each side there is a group of 6-8 small anterior punctiform teeth ... *P. dubius*
- 12b. Buccal armature resembles *P. signatipennis* (above), but the teeth number 34-37 and are very straight and narrow ... *P. occidentalis*
 Buccal teeth 16-20, with a group of 5-6 anterior punctiform teeth at each side ... *P. cinctus*

17. Buccal teeth 50-60, equal and monomorphic 17a
 Buccal teeth less than 40 18
- 17a. Buccal teeth about 50, very delicate, and arranged in an arc deeply concave posteriorly, the outer teeth directed outwardly; some ill-defined nodules at the bases of some of the teeth. Pigmented area with a long anterior extension and a very irregular posterior margin (fig. 66) *P. ruttledgei*
 Buccal teeth 58-60, small and monomorphic, in palisade-formation, straight or only very slightly concave posteriorly. Pigmented area very dark, in the form of a narrow oval, not extending to the lateral walls of the buccal cavity and without any anterior prolongation *P. renauxi*
19. Buccal teeth 35-40 20
 Buccal teeth 25 or less 21
20. Pharyngeal armature very conspicuous (fig. 31). Alar index 0.4-0.8. Epipharynx shorter than AIII. Buccal teeth 35-40, the median teeth being sometimes slightly smaller than the lateral ones. Pigmented area irregularly elliptical, with ragged posterior margin (figs. 52, 53) *P. congolensis*
 Alar index unity. Epipharynx as long as AIII. Buccal teeth 40. Pharyngeal armature less conspicuous than in *P. congolensis* *P. richardi*
21. Buccal teeth equal and monomorphic 23
 Median buccal teeth smaller than lateral ones 22
22. Buccal teeth 13-15, in an arc concave posteriorly. The four lateral teeth on each side very broad, with their points directed towards the mid line, the middle teeth being straight, narrow and parallel (as in *P. decipiens*, fig. 44). Pigmented area elliptical, with a ragged posterior margin and a short anterior process *P. buxtoni*
 Buccal teeth about 20 22a
- 22a. Pharynx markedly constricted in posterior half *P. congolensis* var. *distinctus*
 Pharynx not thus constricted *P. schoutedeni*
23. Buccal teeth 21, in an arc slightly concave posteriorly *P. pastorianus*
 Buccal teeth 25, in an arc strongly concave posteriorly (fig. 54) *P. yusafi*

PROPHLEBOTOMUS MALES

2. Distal segment of superior clasper bearing 4 spines 3
 Distal segment bearing more or less than 4 spines 2a
- 2a. Distal segment bearing only 2 spines *P. mirabilis*
 Distal segment bearing 7 spines *P. hirtus*
3. Buccal teeth vestigial or absent. Pharynx unarmed 3a
 Well-marked buccal armature 4
- 3a. No teeth or pigmented area in buccal cavity *P. wurtzi*
 Buccal teeth vestigial; pigmented area pear-shaped, narrow posteriorly (fig. 64) *P. horgani*
6. Third palpal segment as long as, or longer than, fourth segment:
 Buccal cavity as shown in fig. 38 *P. signatipennis*
 *P. occidentalis*
 *P. dubius*
 Palpal formula 1, 2, 3, 4, 5 7
8. 24-40 teeth in buccal cavity 8a
 Less than 20 teeth in buccal cavity 9

- 8a. Buccal cavity with 25 teeth, arranged in an arc strongly concave posteriorly, the outer 5 teeth on each side broad and each with several points, the central teeth narrow and pointed (fig. 65) ... *P. cowlandi*
- Buccal cavity with 25-35 pointed teeth, arranged in an arc concave posteriorly; median teeth only slightly narrower than lateral ones ... *P. congolensis*
10. Intromittent organ tapering to a sharp point (fig. 19) ... 10a
Intromittent organ tapering to a blunt point (fig. 20) ... 11
- 10a. Palpal formula 1, 2 (3, 4), 5. Proximal segment of superior clasper more than twice the length of the distal segment ... *P. eremitis*
- Palpal formula 1, 2, 3, 4, 5. Proximal segment of the superior clasper less than twice the length of the distal segment, which has the small non-deciduous seta inserted about its mid point (fig. 24).
Buccal teeth 20-60. Pigmented area absent (fig. 37) ... *P. africanus* and vars.
12. Two of the spines on distal segment of superior clasper terminal; the other 2 arise from the middle of the segment. Non-deciduous seta arises proximally to the proximal spines (fig. 23) ... 12a
All 4 spines on distal segment of superior clasper terminal or slightly subterminal ... 13
- 12a. Buccal teeth about 35, long, straight, narrow and pointed, arranged in an arc strongly concave posteriorly, with 2 irregular rows of anterior punctiform teeth ... *P. serratus*
- Buccal teeth about 30, small and rather irregular, arranged in an arc slightly concave posteriorly and having a notch about the centre ... *P. ingrami*
- Buccal teeth about 21, very short and pointed, irregularly arranged in 3 groups—one median, of 4 teeth, and two lateral, of 8-9 teeth.
Pigmented area faint or absent (fig. 70) ... *P. kirki*
16. No posterior row of large pointed teeth in buccal armature, which consists of 4 rows of small punctiform teeth; pigmented area small, oval and ill-defined (fig. 67) ... *P. hunti*
- Posterior row of buccal teeth is either the only row in the buccal cavity or is very large and prominent compared with anterior punctiform ones ... 17
17. Buccal teeth 12-14, markedly unequal, the 4-5 median teeth being pointed and narrower than the lateral ones (as in the female of the same species, fig. 44) ... *P. decipiens*
- Buccal teeth 18-20, not markedly unequal ... 18
18. Buccal cavity rather resembling that of *P. signatipennis* (fig. 38), with a row of small anterior teeth ... *P. babu*
- No anterior teeth. Buccal teeth broad, irregular in contour, and finely crenulated (like the lateral teeth in *P. cowlandi*, fig. 65) ... *P. aretasi*

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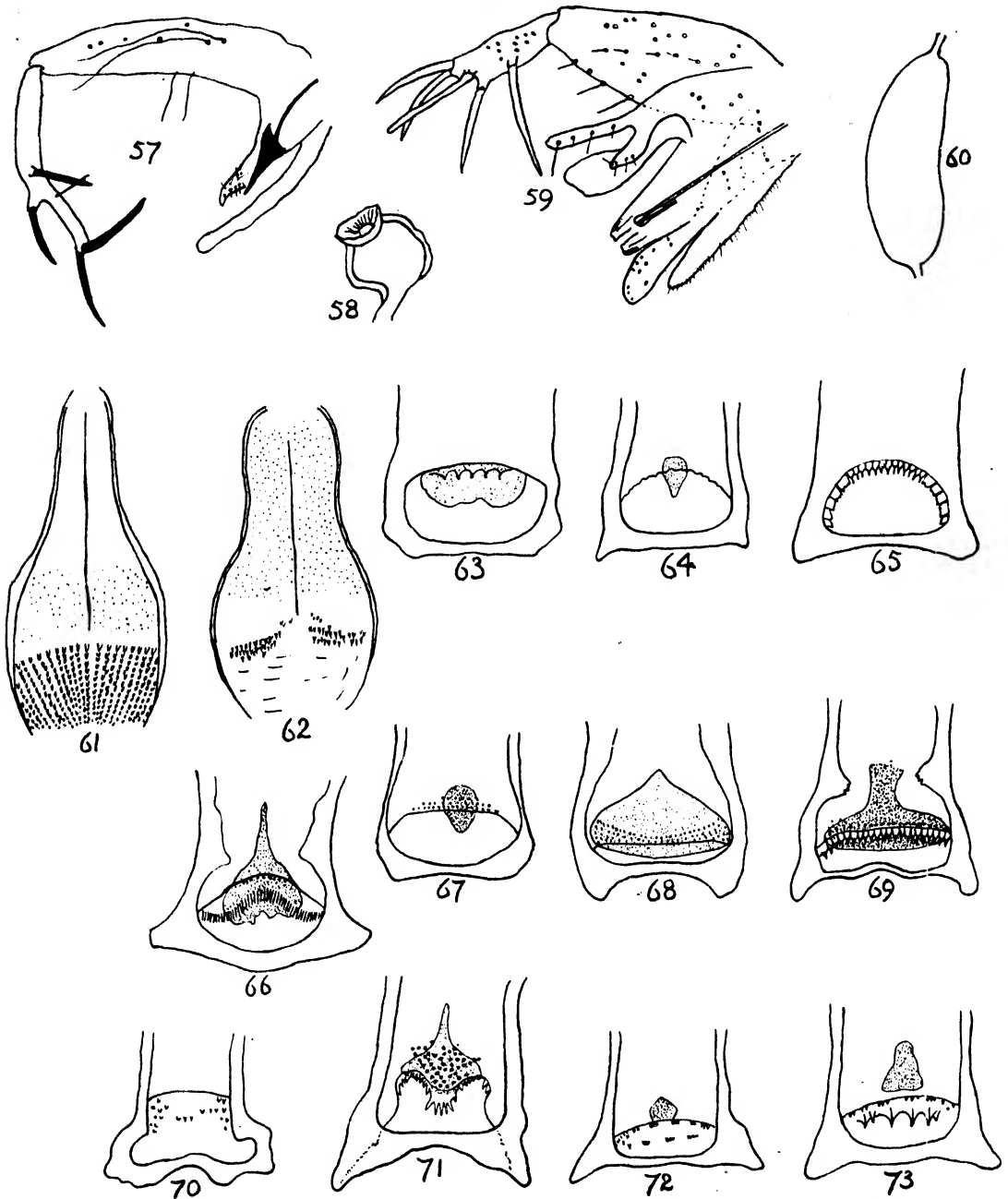


FIG. 57. Terminalia of *P. kirki* (male). FIG. 58. Spermatheca of *P. levisi* (female). FIG. 59. Terminalia of *P. lesleyae* (male). FIG. 60. Spermatheca of *P. lesleyae* (female). FIG. 61. Pharynx of *P. lesleyae* (female). FIG. 62. Pharynx of *P. ruttledgei* (female). FIG. 63. Buccal armature of *P. horgani* (female). FIG. 64. Buccal armature of *P. horgani* (male). FIG. 65. Buccal armature of *P. cowlandi* (male). FIG. 66. Buccal armature of *P. ruttledgei* (female). FIG. 67. Buccal armature of *P. hunti* (male). FIG. 68. Buccal armature of *P. hunti* (female). FIG. 69. Buccal armature of *P. levisi* (female). FIG. 70. Buccal armature of *P. kirki* (female). FIG. 71. Buccal armature of *P. kirki* (male). FIG. 72. Buccal armature of *P. calcaratus* (male). FIG. 73. Buccal armature of *P. calcaratus* (female).

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THE MECHANISMS BY WHICH MOSQUITOES AND TSETSE-FLIES OBTAIN THEIR BLOOD-MEAL, THE HISTOLOGY OF THE LESIONS PRODUCED, AND THE SUBSEQUENT REACTIONS OF THE MAMMALIAN HOST; TOGETHER WITH SOME OBSERVATIONS ON THE FEEDING OF *CHRYSOPS* AND *CIMEX*

BY
R. M. GORDON
AND
W. CREWE*

(From the Department of Entomology and Parasitology,
Liverpool School of Tropical Medicine)

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INTRODUCTION

In addition to the clinical problems sometimes presented by the severe local or, more rarely, generalized reactions of sensitized persons to insect-bites, the feeding-mechanisms of blood-sucking insects, the nature of the lesions produced by their bites, and the subsequent reactions of the human host are of more than academic interest because of their bearing on the transmission and development of the parasites causing insect-borne diseases. Thus the course of an insect-borne disease may be influenced by these factors at any one of three natural phases—that is, at the time when the parasite is taken up by the vector, at the time when it is deposited in the new host, and, finally, during the early stages of its subsequent development.

Let us first consider the taking up of the parasite by a particular species of arthropod feeding on a mammalian host. Here, obviously, the nature of the bite will determine the efficacy of that particular species in extracting a certain species of parasite. To take two well-known vectors of filariasis as examples, the superficial rasping nature of the bite of flies of the genus *Simulium* causes them to take up in large numbers the skin-inhabiting microfilariae of *Onchocerca* spp., whereas the deeply piercing fine mouth-parts of mosquitoes fail to dislodge these larvae, but readily take up blood-inhabiting microfilariae, such as those of *Wuchereria bancrofti*, from the deeper layers. In the latter instance the number of microfilariae taken up is further determined by whether the insect obtains its blood directly from a capillary or from a tiny pool of blood caused by previous laceration of a vessel.

With regard to the depositing of the further-developed parasite in a new host, it is clear that the position of the insect's mouth-parts and the nature of the bite will determine the site and distribution of any parasites introduced by that particular species. Thus the larvae of *W. bancrofti*, which are considered by some authorities to be incapable of penetrating unbroken skin, escape from the labium of the mosquito and are deposited in close apposition to the wound produced by the proboscis, while the supposedly skin-piercing larvae of *Loa loa*, escaping from *Chrysops*, may fall on the skin in a position relatively remote from the abrasion. The medium in which the parasites are deposited

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will also influence their future development. For example, it is known that the *Leishmania tropica* parasites are introduced by the sandfly into the tissues (Shortt and Swaminath, 1928) and, presumably, also into the general circulation. So far as is known, the leishmania parasites introduced into the general circulation are destroyed, while only those at the site of inoculation develop and multiply. In malaria, however, although the feeding mosquito similarly introduces sporozoites into the peripheral circulation and into the tissues, the fate of these, in different species of *Plasmodium*, is not yet clear. We know that in bird malaria, due to *P. gallinaceum*, some of the sporozoites develop at the site of the bite, while in human malaria it appears that only those which reach the internal organs undergo further development. In African trypanosomiasis, as in malaria, it is probable that there is a primary phase of development, but our ignorance of the feeding-mechanism of *Glossina* has prevented our knowing exactly where the metacyclic trypanosomes are deposited. We do know, however, that some of them undergo development at the site of inoculation (Graf, 1937).

Finally, we may consider how the subsequent reactions of the host to the bites of the particular species of arthropod may further influence the introduction of the parasite and its later development. That subsequent reactions do influence the introduction of the parasite is well known. Thus, with the bites of lice and of the reduviid bugs, the introduction of the saliva into sensitized persons results in irritation and scratching, which facilitates the entry of the parasites and so greatly increases the risk of infection with typhus or South American trypanosomiasis. On the other hand, we are almost completely ignorant of the effects on the introduced parasite of any local reactions of the host to the secretions of feeding insects, although it is quite possible that such reactions may alter the development of any introduced protozoan or metazoan parasites which undergo a primary phase of development at the site of inoculation.

As a preliminary to studying this primary phase of development of certain parasites after their injection by blood-sucking flies, we undertook the experiments with *Aedes*, *Glossina* and *Chrysops* described in this paper. We also made a few observations regarding *Cimex*, for, although the bed-bug is not known to transmit infection in nature, we thought that it would be of interest to compare the lesions produced by one of the Hemiptera with those caused by certain blood-sucking Diptera.

It is, of course, well known that severe generalized allergic manifestations may occur in sensitized human subjects (Hecht, 1930) or in animals (Ciurea and Dinulescu, 1924) following the injection of unusually large doses of insect antigen. We have observed such reactions in sensitized persons after exposure to the bites of several hundred mosquitoes; but, since our main object was an investigation of the local reactions following insect-bites, with a view to future study of their possible effects on the subsequent development of any introduced parasites, we did not otherwise concern ourselves with these general reactions.

GENERAL REVIEW OF THE LITERATURE

At this stage it would appear reasonable to summarize, in very general terms, the views of various authors on the mechanism and effects of insect-bites. For the most part, the descriptions of these authors of the mechanism of the mouth-parts are founded on anatomical studies, and, since observations during the actual process of biting are scanty and incomplete, it must be concluded that their interpretations of the movements and function of the mouth-parts, although probably correct, are mainly conjectural. It is

also true that our knowledge of the effects produced is founded rather on clinical manifestations in sensitized and non-sensitized persons than on exact knowledge derived from histological study.

The literature concerning the bites of those insects in which we are interested is widely distributed, but the generally accepted views, as summarized in text-books of medical and veterinary parasitology, may be expressed somewhat as follows. The insect cuts the skin, causing the minimum of damage, and into the wound thus made it inserts its proboscis and injects saliva into the tissues of the host. The proboscis is a more or less rigid structure, and the insect inserts it to an increasing depth until it encounters a blood-vessel, from which it feeds. Having finished its meal, the insect withdraws the proboscis along the same path by which it entered, so that, as a result of this very fine instrument being inserted along a single track, practically no trauma and very little haemorrhage into the tissues is produced. As regards the subsequent reaction, it is generally thought that the bites of these insects normally produce some reaction, although the intensity of this reaction varies according to the individual's previous history of exposure. Thus some persons become increasingly sensitive to the bites of certain species, whilst others develop an immunity and eventually cease to react. In addition to this visible reaction, it is accepted that some degree of itching is associated with the bite, the intensity varying proportionately with the visible reaction.

Our own observations, the results of which are recorded below, differ from this conception in certain important details, particularly concerning the rigidity of the proboscis, the source from which the insect obtains its blood-meal, the nature and extent of the lesions produced, and the causes of the subsequent reaction.

SCOPE OF THE INVESTIGATION

As previously stated, our main object during this work was to investigate the medical significance of the reactions of the mammalian host to arthropod bites, and particularly the possible effects of such reactions on the transmission and early stages of development of introduced parasites causing insect-borne diseases.

The following account is divided into two parts, Part I being concerned with the feeding-mechanism of mosquitoes and with the lesions produced by their bites, and Part II with the bites of tsetse-flies. Sensitization was observed to occur in man following the bites of all the species of insects with which this paper is concerned, but, whereas the histology of the lesions in sensitized persons and the passive transfer of such sensitization to animals were studied only in relation to *Glossina*, the clinical aspects of sensitization, as influenced by regular and irregular feeding, were investigated mainly in persons bitten by *Aedes aegypti*. It would, of course, have been preferable to study the clinical and histological manifestations of sensitization fully in both insects, but practical difficulties prevented this. We have, however, no reason to suppose that these aspects of sensitization will prove to differ markedly in the different insects.

PART I

THE FEEDING-MECHANISM OF THE MOSQUITO AND THE LESIONS AND REACTIONS PRODUCED BY ITS BITE

Most of our experiments were carried out with a strain of *A. aegypti*, but for comparison the lesions following the bites of *Anopheles maculipennis* were also studied, and

various species of mosquitoes were employed as controls in the sensitization and desensitization tests. The histology of the lesions produced by the bites of mosquitoes was studied in laboratory animals, but the clinical study of sensitization and desensitization only in the human host. It is generally agreed that animals are difficult to sensitize to insect-bites, and, although Dubin *et al.* (1948) succeeded in sensitizing rabbits, we have never observed sensitization to follow the feeding of a variety of insects on our laboratory stock, even when such feeding was of a very irregular character. We failed in our attempts to transfer

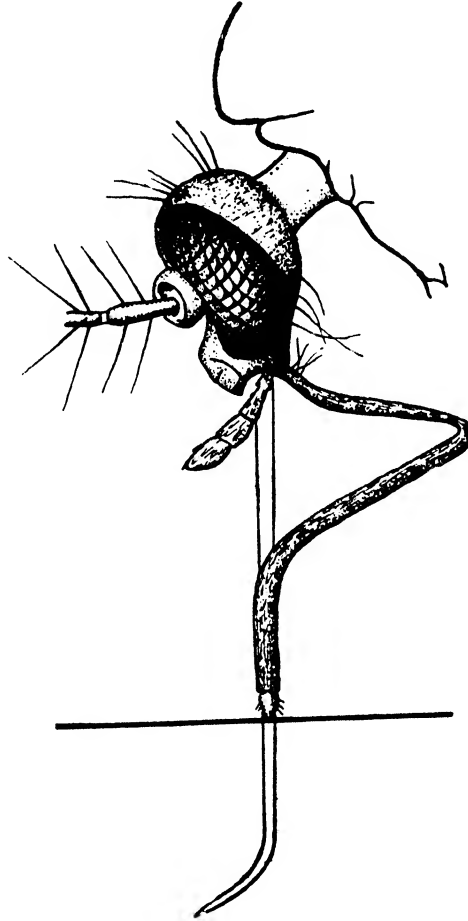


FIG. 1. The curving of the fascicle of *Aedes aegypti* as observed in frog's web. (Semi-diagrammatic; $\times 35$.) (After Gordon and Lumsden, 1939.)

passive sensitization to rabbits by the intradermal injection of serum from persons sensitive to mosquitoes, although, as later recorded, we were successful when using the serum of persons sensitized to *Glossina*.

The difficulty of recording dermal reactions for comparison at later dates is generally recognized, and we found that the best method was by the use of colour photography. The colour photographs, the reproduction of which we consider unnecessary, are the standards by which we compared the reactions described below.

THE FEEDING-MECHANISM

There are numerous descriptions of the feeding-mechanism of mosquitoes, notably the anatomical study by Robinson (1939) and the observations of Gordon and Lumsden (1939) on the behaviour of the fascicle as observed in living tissue. Our more recent observations have generally confirmed Gordon and Lumsden's account, which may be summarized as follows. The insect alights on the skin, probes a few times in preliminary inspection, and commences to feed. Throughout the process of feeding the labium remains outside the skin, with the labella pressed closely together. The mandibles and maxillae cut the skin, and into the wound thus made is thrust the proboscis, consisting of the labrum and hypopharynx, the mandibles and maxillae. The mandibles and maxillae proceed to cut the tissues, and thus allow the forward thrust of the whole fascicle. The distal portion of the fascicle is actively flexible, so that it can be curved—often in the form of a J—to an extent allowing of penetration in almost any direction, while the remainder of the fascicle is passively flexible, enabling it to accommodate itself to the curves previously pursued by the cutting tip (fig. 1).

THE LESIONS PRODUCED

We have failed to trace any previous description of the histology of the skin areas bitten by mosquitoes, but Gordon and Lumsden (1939) observed the behaviour of the proboscis during the act of feeding in the transparent membrane of frog's web, and came to the conclusion that the mosquito took up blood by two distinct methods. In the first, which has already been referred to as the generally accepted view, the labrum enters the lumen of a capillary and the blood is sucked up directly from the vessel (fig. 2). In the second, which had not previously been described and to which they refer as 'pool feeding,' blood is taken up from an extravasation derived from a previously lacerated capillary (fig. 3). They further showed that fluid—presumably salivary secretion—was injected into the tissues at various stages of penetration of the proboscis.

The present series of observations were made after feeding *A. aegypti* on marked areas on guinea-pigs previously anaesthetized with nembutal. The guinea-pig was killed either one hour or 24 hours after the bite, and the marked areas of skin were removed, usually to a depth to include the underlying muscle. Serial sections of the tissues so obtained were then stained and examined.

Study of these sections has confirmed the observations already referred to. In tissues removed one hour after biting, small, usually localized, haemorrhages were, in certain instances, observed in the corium, and it appears reasonable to presume that these were the result of pool feeding (fig. 4). In other instances no abnormalities were present, presumably because feeding had taken place directly from the lumen of a capillary. In tissues removed 24 hours after feeding, distinct, and often larger, haemorrhages were present, and occasionally there was also a distinct swelling at the site of the bite, which was seen to be caused by separation of the collagen fibres (fig. 5). This swelling could not have been due to any sensitization of the animal, since those used for histological examinations had not previously been exposed to mosquito-bites, and since the reaction was not observed in tissues removed one hour after biting. The swelling thus appears to correspond with the delayed reaction well known to occur in man. This histological evidence of a delayed reaction in animals is of some interest, since it was never sufficiently marked to be obvious to the naked eye, and until we observed it microscopically we were

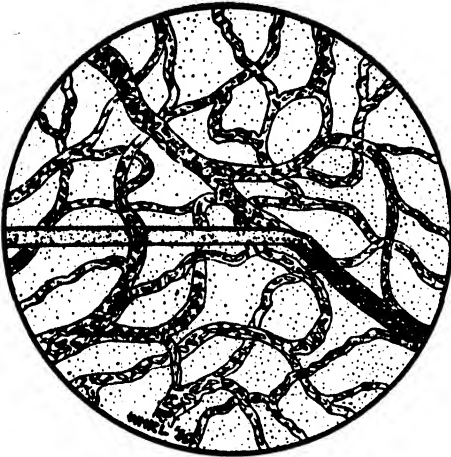


FIG. 2. Capillary feeding of *Aedes aegypti*. The tip of the fascicle lies in the lumen of a capillary.

(Semi-diagrammatic; \times about 70.)

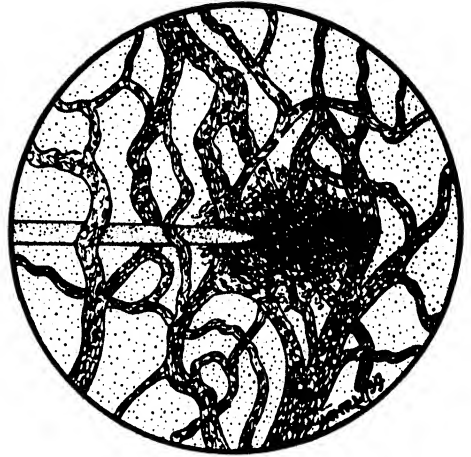


FIG. 3. Pool feeding of *Aedes aegypti*. The lacerated capillary is bleeding into the tissue spaces, and the extravasated blood is being sucked up the fascicle.

(After Gordon and Lumsden, 1939.)

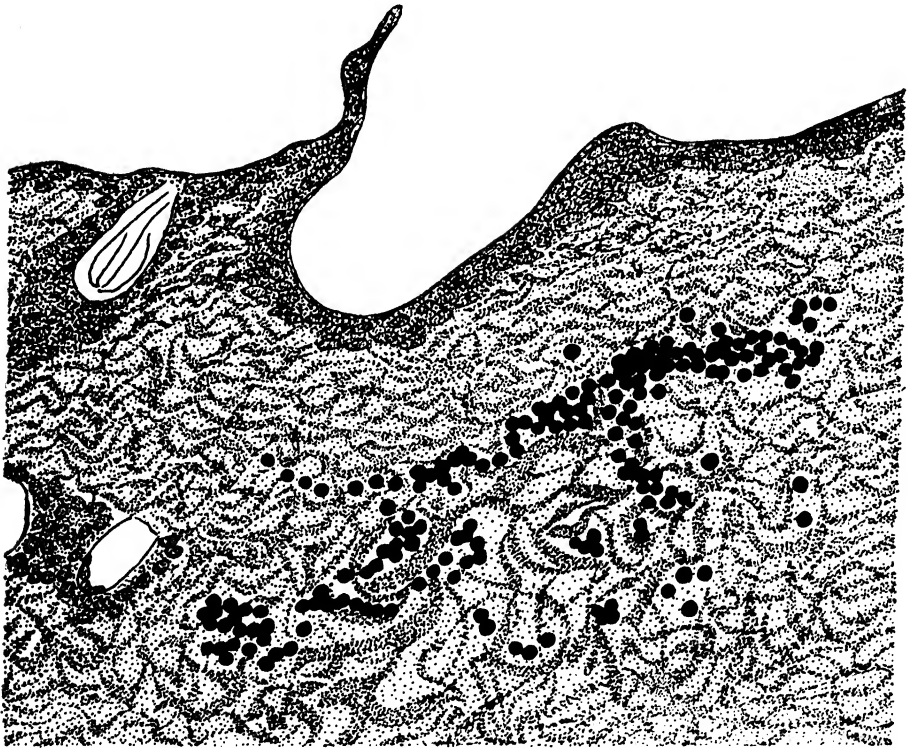


FIG. 4. Section of skin of a guinea-pig, removed one hour after bite of *Aedes aegypti*, showing a small haemorrhage. (\times 400.)

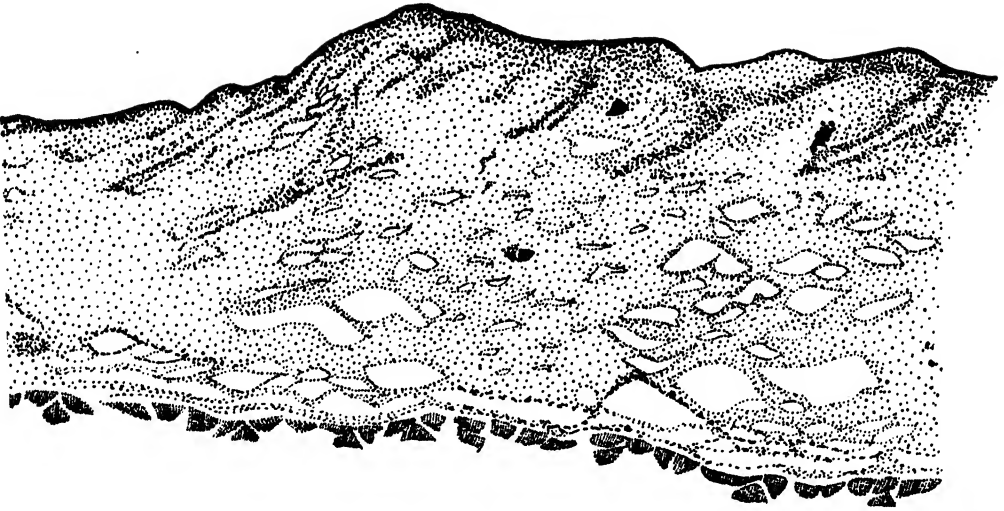


FIG. 5. Section of skin of a guinea-pig, removed 24 hours after bite of *Aedes aegypti*, showing small localized haemorrhages and local swelling caused by separation of the collagen fibres. ($\times 80$.)

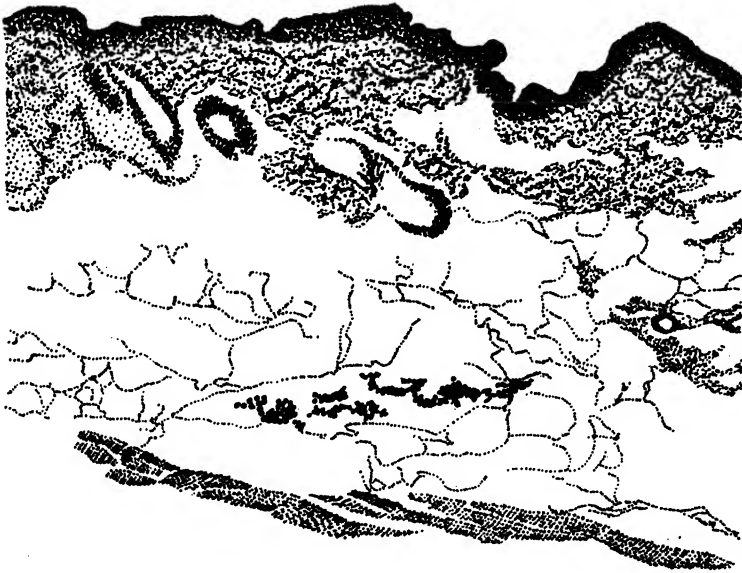


FIG. 6. Section of skin of a guinea-pig, removed one hour after bite of *Anopheles maculipennis*, showing a small haemorrhage in which the red cells are agglutinated. ($\times 200$.)

under the impression that laboratory animals exposed to mosquito-bites failed to develop any reaction.

It would appear, then, that the mosquito, by means of its flexible proboscis, probes the tissues in many directions, and that in non-sensitized animals, and presumably in non-sensitized humans, the only immediate effect of the bites of *A. aegypti* is the presence of one or more slight haemorrhages, which persist for at least 24 hours, during which time there may be an escape of fluid from the capillaries amongst the collagen fibres, causing a swelling known as the delayed reaction. We observed no abnormality of the shed red cells, but these tests were carried out with *A. aegypti*, the salivary secretion of which is not known to contain any agglutinin or haemolysin, or any substance affecting coagulation. In the case of *A. maculipennis*, on the other hand, it is known that an agglutinin exists in the salivary secretion (Yorke and Macfie, 1924), and in tissues removed from a guinea-pig shortly after it had been bitten by this insect agglutination of the red cells in the haemorrhages was well marked (fig. 6).

THE IMMEDIATE AND THE DELAYED REACTIONS

The subsequent reaction to the bites of *A. aegypti*, and apparently to the bites of all mosquitoes, may be one of two kinds (Hecht, 1943; Mellanby, 1946a): either an immediate wheal reaction, which is exhibited by a limited number of persons, or a delayed papular reaction, which, although it varies in intensity, appears to be almost universal.

The Immediate Reaction

Nature of the immediate reaction. The explanations given in the literature of the cause of the immediate reaction are various, but fall into three categories. Firstly, the insect, during the act of feeding, introduces into the tissues a poison, often referred to as a 'toxin,' which causes the wheal associated with the bite; secondly, the insect introduces a substance which, although in itself harmless, yet if repeatedly introduced at irregular intervals causes a state of allergy, so that the individual becomes 'sensitized' and reacts violently to the introduction of the same substance as a result of further bites; thirdly, there is a combination of these two causes.

The first view, as a complete explanation of the reaction, is now generally regarded as obsolete, although it is still quoted in various text-books. For example, by MacLeod and Muende (1946) it is said that the bed-bug, 'On puncturing the skin with its stylet, . . . injects an irritating salivary fluid, which gives rise to an urticarial lesion with a central bleeding-point.' Andrews (1946) says that 'Mosquitoes and gnats inject an irritating fluid under the skin before sucking the blood and this provokes a pruritic urticarial wheal or less often a blister, a diffuse swelling or lymphangitis.' On the other hand, although most authorities accept the view that marked reactions are due to sensitization (e.g., Hecht, 1928, etc.; Fairley, 1936; Benson, 1939), there appears to be a general belief that biting insects usually introduce some poison, so that persons when bitten for the first time react immediately, even though mildly. This was our own impression when we began the series of experiments which we are describing; but as a result of our investigations we were led to the belief that, in the insects with which we experimented, no such rapidly acting toxin is introduced. It is difficult to prove this in the case of mosquito-bites in general, since most persons have been bitten at one time or another; but in the case of

anophelines, to which persons in this country are relatively seldom exposed, it is unusual to find such individuals reacting immediately on the first occasion of exposure—a fact which has been pointed out by Mellanby (1946a) and others.

Factors influencing the production of the immediate reaction. It is well known that the injection at irregular intervals of varying amounts of a protein tends to sensitize the individual to that particular protein, whereas allergy does not usually develop if the protein is injected at regular and frequent intervals in similar amounts. In order to prove whether the immediate reaction is due to specific sensitization or to the injection of a generally toxic substance, we observed the reactions in man following exposure to regular and irregular feedings of *A. aegypti*.

As regards reaction to irregular feeding, it is usual to find that technicians working in the insect-rooms, who are not deliberately feeding *Aedes* on themselves but are exposed to occasional bites from escaped insects, become sensitized. During the course of the present investigation we have had the opportunity of observing such an individual, who has been exposed to numerous bites of *Aedes* at irregular intervals over a period of three years. This person became sensitized within a few weeks of first being bitten by the mosquitoes, and is still very sensitive, despite having been exposed to *Aedes* a great number of times. Another individual also became sensitive to the bites of *A. aegypti* shortly after the first bites, but after further exposures ceased to react. These observations suggested that the immediate reaction was due to sensitization.

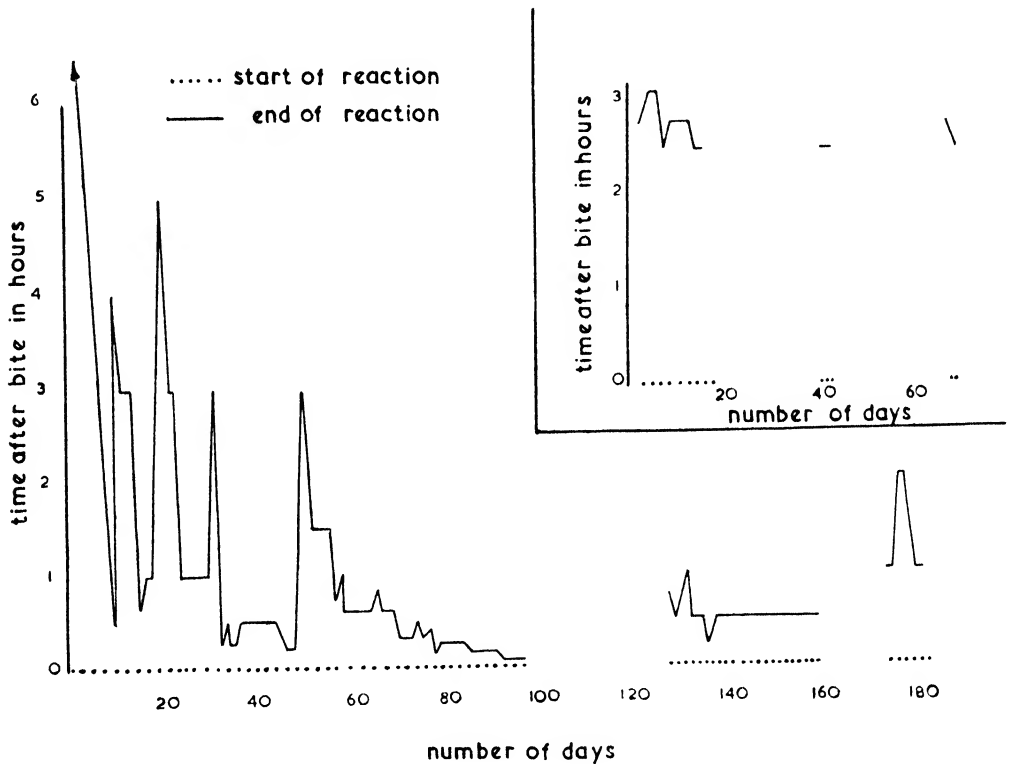
Since the intensity of the reaction varies proportionately with the amount of fluid injected, it is often thought that the immediate reaction to insect-bites is due to the injection of a toxin. On further investigation, however, the reacting person will usually be found to have a previous history of sensitization, while it has been demonstrated that non-sensitized individuals do not show an immediate reaction even though exposed to the bites of several hundred mosquitoes at the same time.

To determine whether sensitization would follow regular feeding, we observed an individual who had not previously been exposed to *A. aegypti*, and who appeared particularly well suited for our purpose, since he was easily sensitized to the bites of other insects, having in turn become sensitized to the bites of *Glossina* and *Haematopota*. This individual was exposed regularly, almost daily, to the bites of *A. aegypti*, and experienced 384 bites within 185 days. No sensitization followed this exposure, although when tested with *Glossina* he still reacted violently to the bite of this insect.

Factors influencing the disappearance of the immediate reaction. If the immediate reaction is due to sensitization, one would expect it to persist in the case of irregular feeding and to tend to disappear with regular feeding. That this sometimes occurs in nature has been noted by Martini (1925, 1926) in the case of woodland species of *Aedes*, and that it can be reproduced experimentally is shown by the behaviour of the two sensitized individuals whose reactions are plotted in graph 1. Case 1, who was exposed to two or three bites at irregular intervals over a period of 70 days, was not desensitized, while case 2, who was regularly exposed to two or three bites daily, was almost completely desensitized at the end of the same period, and remained desensitized so long as regular feedings were continued—in this case, for a further period of 26 days. That, at least in this individual, desensitization is not permanent is shown by the fact that he subsequently reacted more violently during each of two periods after regular feeding was discontinued. The transient nature of desensitization to mosquito-bites has been noted by Benson (1939), while Mail

(1934) reported the return of sensitization following a cold treated with large doses of citro-carbonate of magnesia.

Our experiments on sensitization and desensitization were conducted with live insects, and we made no observations on the effect of vaccines prepared from the insect's salivary glands. The possibility of desensitizing susceptible persons by the use of antigens prepared from the species of insect responsible has been investigated by Benson (1936) for mosquitoes, and, for cases of sensitization to flea-bites, by Cherney *et al.* (1939), by McIvor and Cherney (1941, 1943), and by Hatoff (1946). As a result of their experiments



GRAPH 1. Showing the effect of regular and irregular exposure to the bites of *Aedes aegypti* on the immediate reaction in two individuals previously sensitized to this species. The inset shows how the reaction is sustained by irregular feeding (case 1), while the main graph shows the gradual desensitization produced by regular feeding and the return of sensitization after a period during which no bites were experienced (case 2).

these writers considered that the administration of the specific antigen to sensitized persons was beneficial, the wheals produced by subsequent bites being smaller in size and persisting for a shorter period. On the other hand, McKinley (1929) failed to desensitize with salivary-gland extracts persons sensitive to the bites of *A. aegypti*.

Specificity of the immediate reaction. The specificity of the reaction to insect-bites has been discussed by a number of writers: by Boycott (1912, 1926, 1928) for fleas and sandflies, by Hecht (1928) for mosquitoes, fleas and bed-bugs, by Hase (1929) for *Cimex*, and by Mellanby (1946a) for mosquitoes. How selective the response to a particular salivary secretion may be was shown as long ago as 1912 by Boycott, who observed that

persons sensitive to *Xenopsyllus cheopis* were not sensitive to *Nosopsyllus fasciatus*; and a still more remarkable selectivity is quoted by Hase (1929) in the case of persons who reacted differently to *Cimex lectularius* and *C. tropica*.

Boycott (1928) has pointed out that 'It is difficult to make satisfactory experiments with our native insects since we can never be sure whether anyone has or has not been bitten before by any particular species of "gnat" or "midge".' It appeared to us that a somewhat more accurate approach to the problem of specificity might be obtained by selecting an individual who reacted strongly to two related species of mosquito, desensitizing him to one species by repeated regular feeding by that species, and subsequently testing his reaction to the other. Case 2, as a result of feeding *A. aegypti* and *Culex molestus* at irregular intervals, became markedly, and about equally, sensitive to the bites of both insects. He was then exposed only to *A. aegypti*, which were fed on him daily, with few omissions, for a period of 62 days. At the end of that period he was almost completely desensitized to *A. aegypti* but still reacted as strongly as originally to *C. molestus*.

Effect of drugs on the immediate reaction. It has been stated by Shannon (1943) that the oral administration of thiamine chloride (vitamin B₁ hydrochloride) diminished the intensity of the reaction to insect-bites subsequently received—an observation which was confirmed by Eder (1945) but not by Wilson *et al.* (1944). If, as we believe to be the case, an immediate intense reaction following mosquito-bites is due to sensitization, the swelling and redness will partly be due to the release of histamine, causing dilatation and increased permeability of the capillaries. To determine whether the anti-histamine drug benadryl would reduce the reaction, two individuals highly sensitive to the bites of *Aedes* were each given 150 mgm. of benadryl daily for three days, and on the second day were exposed to the bites of *A. aegypti*. No appreciable reduction was observed in the immediate reaction, as shown by the comparison of colour photographs taken before and during the benadryl course, but a marked diminution of the itching was recorded in both instances. This failure of benadryl applies only to the local reaction, and there is evidence to suggest that the drug does reduce the general reaction following the injection of large amounts of salivary antigen into a highly sensitive person.

Localization of the immediate reaction. It is sometimes stated (e.g., by Herscheles, 1922) that persons are 'more immune' in one area of the body than in another, a common observation being that the arm which is more frequently used to feed a particular insect does not react so violently as the other arm which is less often employed. We believe that it would be more accurate in such circumstances to say that one arm is more desensitized than the other, for the fact that an individual reacts strongly at all is, in our opinion, evidence that he has at one time become sensitized. We have carried out no experiments on sensitizing an individual by irregularly feeding mosquitoes on a localized area of the body, and therefore do not know whether sensitization to mosquitoes is at first localized or generalized. On the other hand, case 2, who was originally sensitized to the bites of *A. aegypti*, was desensitized by regular feeding on one arm, and we observed that during the period of desensitization he reacted less severely on that arm than on other areas of the body, thus indicating that desensitization, at least, is to some extent localized.

The source of the substance producing the immediate reaction, and the method of its injection. There appears little doubt that the injection of salivary fluid is responsible for the reaction and for the irritation associated with the bite, for, although Schaudinn (1904), Hindle (1914) and Roxburgh (1927) have suggested that the swelling and irritation

are due to the regurgitated contents of the oesophageal diverticula, the work of later writers, such as Hecht (1928), Pawlowsky and Stein (1928) and Manalang (1931), proves that, in nature at least, it is the salivary secretion alone which is responsible. There also appears to be considerable evidence that the responsible substance is injected throughout the act of feeding, and is therefore widely distributed in the tissues, for we have repeatedly observed that the intensity of the reaction is directly proportional to the length of time during which the mosquito is allowed to feed. This latter fact was also noted by Hecht (1929).

At this stage we may briefly summarize the results of our observations concerning the immediate reaction to the bites of *A. aegypti*. The immediate reaction commences during, or immediately after, the act of biting, and takes the form of a wheal, often with well-marked pseudopodia, and surrounded by an area of erythema. The substance causing the reaction is contained in the salivary fluid, and is injected into the tissues by the mosquito throughout the act of feeding. It is in itself incapable of causing a reaction, and in non-sensitized subjects the only immediate histological lesions are due to the mechanical trauma caused by the insect's mouth-parts; but if it is injected repeatedly at irregular intervals it causes a state of allergy in the host, so that further injections of the same substance will produce the reaction. If the injection of this substance at irregular intervals is continued, the state of allergy persists, but if it is injected at regular and frequent intervals the reaction steadily diminishes in duration and intensity, until the individual is desensitized and ceases to react. The sensitizing substance is highly specific, so that an individual may be sensitive to the bites of one species of mosquito and, at the same time, insensitive to another.

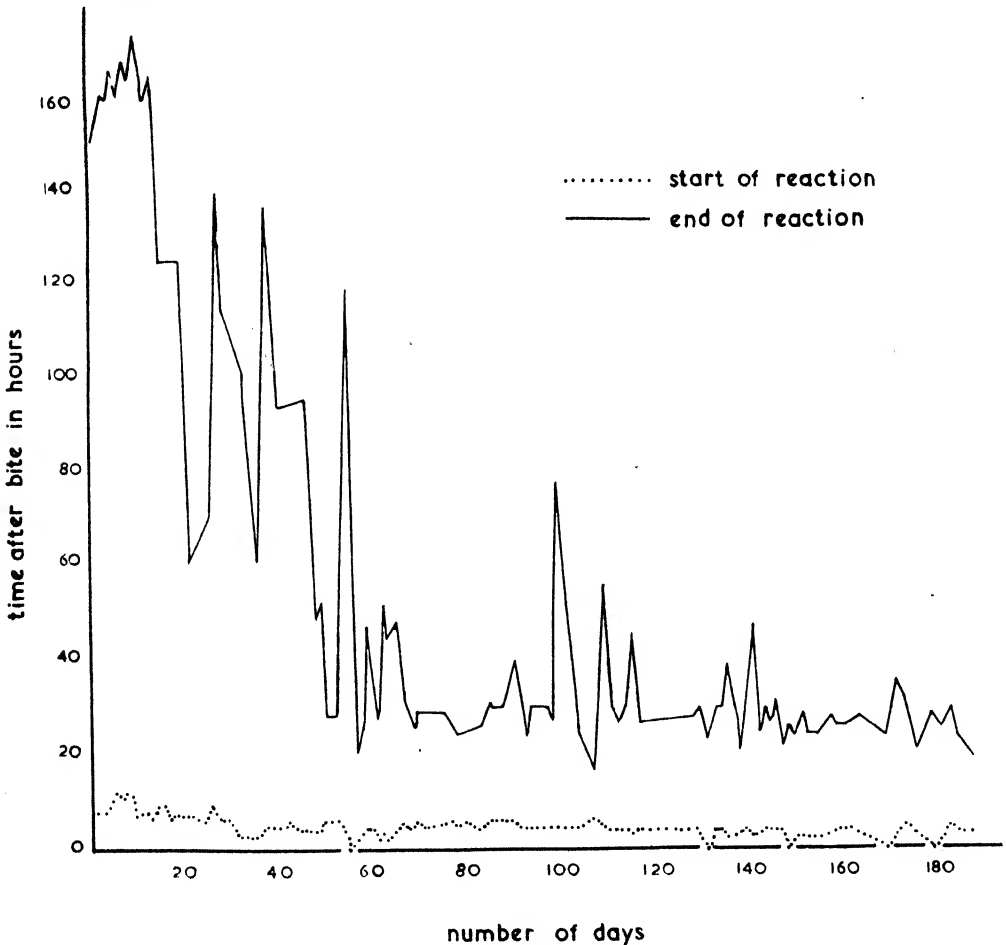
The Delayed Reaction

We have already drawn attention to the fact that man's reactions to the bites of mosquitoes are of two kinds: an immediate reaction, which generally commences while the mosquito is biting, and a delayed reaction, commencing a variable period (usually about 24 hours) after a bite. Our observations on the delayed reaction were conducted on similar lines to those previously described on the immediate reaction, but since they were less extensive we propose to consider them more briefly.

Theodor (1935), working with *Phlebotomus*, suggested that the delayed reaction was experienced at an early stage of sensitization, and that further biting reduced the latent period until the reaction became immediate. Mellanby (1946a), however, considered that, in the case of mosquito-bites, the two reactions were probably caused by what he referred to as 'different antigens' in the saliva, basing his opinion on the fact that both the reactions could occur in the same individual as the result of the same bite. Our own observations confirm that the two reactions, although both produced by the salivary secretion, have separate causes; but they suggest that, whereas the immediate reaction is due to the injection of a substance to which the individual has previously been sensitized, the delayed reaction is caused by the injection of a slow-acting poison which produces its effects independently of previous biting, and is therefore more common. This universality of the delayed reaction is not usually obvious, since in our experience persons seldom complain of, or even notice, the delayed reaction, although Mellanby (1946b) states that the delayed reaction is 'more troublesome.'

Mellanby (1946a) describes how 25 volunteers, who had never been out of this country,

were exposed to the bites of *A. aegypti* and each developed a delayed reaction but no immediate reaction. During our experiments we have noticed that the delayed reaction occurred following the bites of various species of mosquitoes, and that in all cases the reaction developed after the first few bites, if not after the first bite, of a species to which the individual had not previously been exposed. Hecht (1943) states that, following persistent biting by *X. cheopis*, he observed a late reaction in persons who had previously



GRAPH 2. Showing the effect of regular exposure to the bites of *Aedes aegypti* on the delayed reaction in an individual not sensitized to this species. The duration and intensity of the reaction gradually diminish, and the start of the reaction becomes progressively earlier.

shown only an immediate reaction, but he does not explain whether the delayed reaction was newly developed or only became observable as a result of the shortening of the duration of the immediate reaction caused by the persisting feeding.

It is well known that the delayed reaction tends to disappear in individuals who are exposed over long periods to the bites of the insect concerned, and it is of some interest to consider the reasons for this acquired 'immunity,' which, since the reaction is not due

to sensitization, cannot be due to desensitization. Whereas irregular exposure to insect-bites produces and maintains the immediate reaction, our own observations and the evidence of individuals exposed to mosquitoes for many years, either in the tropics or in this country, show that irregular exposure over long periods causes the disappearance of the delayed reaction.

Regular exposure, on the other hand, causes the disappearance of the immediate reaction, and, in order to test its effect on the delayed reaction, we observed an individual who showed a delayed reaction after his first exposures to *A. aegypti*, and was then exposed to two or three bites daily, with few omissions, for 185 days. Under these conditions sensitization did not develop, and freedom from the delayed reaction was progressively acquired, the reaction gradually becoming of shorter duration and its intensity becoming so slight that the reaction was scarcely noticeable (graph 2). Apart from this decrease in duration and intensity, the onset of the reaction became progressively earlier, so that towards the end of the period of observation it sometimes occurred immediately after the bite. In spite of its early appearance, however, it in no other way resembled the severe immediate reaction following sensitization, since the intensity had become so slight that the reaction would have passed unnoticed unless specially looked for.

Our observations on the delayed reaction may be summarized as follows. The substance responsible, as in the case of the immediate reaction, is contained in the salivary fluid and is injected into the tissues by the mosquito throughout the act of feeding. The delayed reaction is normally far less severe than the immediate reaction; it is papular in appearance, and does not show the wheal with pseudopodia characteristic of the immediate reaction. It may, and commonly does, appear independently of previous exposure to the bites of the insect concerned, while, unlike the immediate reaction, it disappears after irregular as well as after regular feeding. These observations suggest that the reaction is due, not to sensitization, but to the injection of a slow-acting poison. Histologically, the resultant swelling is seen to be due to separation of the collagen fibres, presumably caused by leakage of fluid from the capillaries. The small haemorrhages often present are apparently caused by the mechanical action of the insect's mouth-parts.

Our laboratory observations on the non-appearance of the immediate reaction and the gradual disappearance of the delayed reaction after prolonged regular feeding are confirmed by the fact that young children in areas densely infested with mosquitoes tend to react to bites more strongly than adults (Martini, 1925, 1926), and suggest that the 'immunity' to mosquito-bites generally exhibited by natives of all ages in the tropics is not a racial characteristic, but is due to the fact that from birth they are regularly exposed to the bites of these insects.

PART II

THE FEEDING-MECHANISM OF THE TSETSE-FLY AND THE LESIONS AND REACTIONS PRODUCED BY ITS BITE

The flies used for this part of the work were bred from pupae sent to us at about fortnightly intervals from trypanosomiasis research centres in East Africa. Most of the pupae were those of *Glossina palpalis*, but a few batches of *G. morsitans* were also received. Generally we used *G. palpalis* for our experiments and identified each fly before using it; but on occasion this examination was omitted, so that we cannot state with certainty the species used in every experiment.

THE FEEDING-MECHANISM

The feeding behaviour of *Glossina* is described by Lester and Lloyd (1928) as follows. 'Resting on the skin the fly suddenly braces itself, spreading its legs a little. The palps stick straight out while the proboscis is lowered at right angles and introduced into the skin. If blood is not obtained quickly the fly pushes its proboscis in up to the bulb and moves it up and down, lacerating the tissues. If this does not cause a flow of blood the fly moves a little way on the skin and tries again till a satisfactory source of blood is found. It then feeds with about one-third of its proboscis inserted.' To this description we might add the following additional points derived from our own observations. The fly never appears to feed after the first probe, when the proboscis is inserted until the bulb almost touches the skin. The proboscis is then partly withdrawn and reinserted deeply, the head being almost invariably moved, so that the subsequent downward thrust is in a different direction from the previous one. This cycle is repeated a few, or sometimes many, times until the fly finally settles down and feeds. Once feeding has begun, distension is very rapid the abdomen becoming swollen with blood in about a minute; it is this rapid filling with blood which has probably led to the belief (e.g., by Rodhain *et al.*, 1912) that the fly must be feeding directly from a vessel.

Previous interpretations of the subsequent movements and of the function of the mouth-parts are based on anatomical studies of the proboscis outside the host tissues. Of these studies, that of Jobling (1933) is outstanding. It is difficult to condense his careful and accurate account, or even to describe his conclusions, without reference to the numerous figures illustrating his paper, but the following would appear to summarize his views concerning the behaviour of the mouth-parts during the act of feeding. The haustellum is a very slightly curved, but more or less rigid, rod mounted proximally on a loose ball-and-socket joint. When at rest it lies horizontally between the palps, but it may be depressed and directed downwards, as occurs when the fly is feeding. Besides the depression and elevation, which appear to be the main movements, the haustellum is capable of limited retraction and of movement from side to side. In the retracted position, the armature is withdrawn and lies concealed within the labella, but in the everted position the outer walls of the labella may contract and evert the inner walls and the armature. During the retraction and protraction of the outer walls, the inner walls become alternately everted and inverted, and since the armature is attached to these walls its teeth perform protrusible divergent movements, which serve for cutting the skin.

The technique of observing the proboscis in the transparent membrane of frog's web was found to be inapplicable to the tsetse, and we therefore studied the behaviour of the proboscis in the tissues by an indirect method. The fly was allowed to feed on an anaesthetized guinea-pig, and the proboscis was cut off very close to the head of the fly while blood was being withdrawn. The severed proboscis was fixed in position with a drop of celloidin, the guinea-pig was killed, and the tissue containing the proboscis was removed, fixed and cleared. The resultant block was then trimmed, either on a microtome or by hand, until the course of the proboscis was visible in the now cleared tissues.

The observations thus made have confirmed the extensive anatomical studies of Jobling in all but the following points. In our sections showing the proboscis in the tissues we have not observed complete eversion of the armature. However, as it shown in Plate XII, fig. 1, partial eversion, with consequent protrusion of the teeth, does occur, thus indicating that the alternate complete eversion and inversion of the armature may

take place. Secondly, Jobling regards the proboscis as flexible only at the point where the theca joins the labial gutter, and considers that this flexible portion of the haustellum is straightened and strengthened during feeding by the interaction of the retractor and protractor muscles of the theca. Our observations prove that the whole haustellum is a flexible structure, which may be curved during the act of piercing the skin, either in the same vertical plane as that in which it passes through the skin or at an angle to that plane (fig. 7). This again is not a complete contradiction of Jobling's view, since it appears to us that muscles which are capable of holding the haustellum straight are equally capable of holding it in a fixed curve.

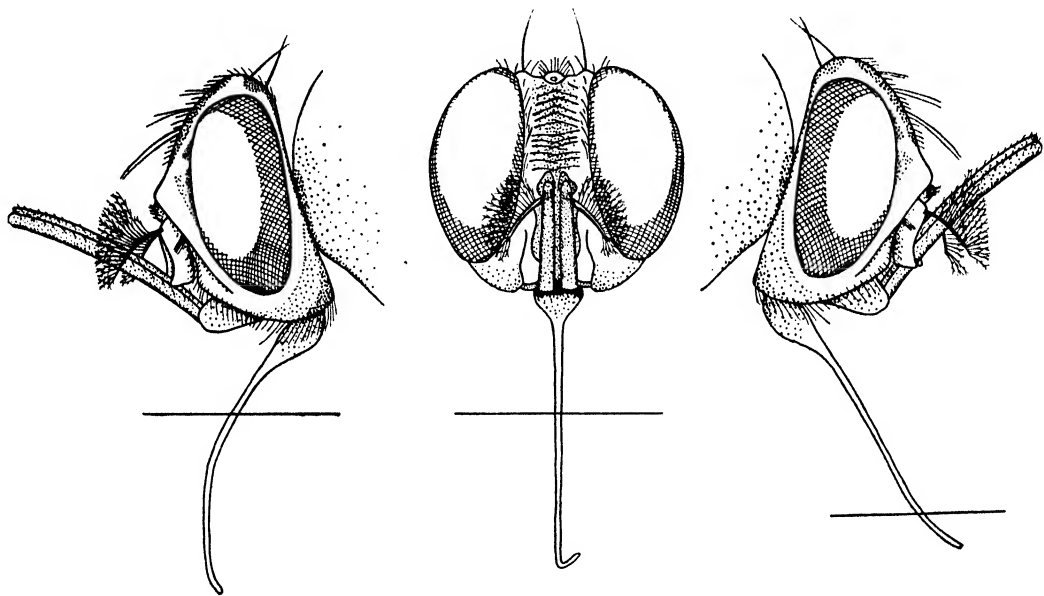


FIG. 7. Diagrammatic representation, prepared from camera-lucida drawings, of three of the various positions assumed by the proboscis of the tsetse-fly during the act of feeding in living tissue. (\times about 14.)

From our observations it would appear, then, that the proboscis of the tsetse, like that of the mosquito, is a flexible structure which the insect repeatedly thrusts into the tissues of the host along different paths. Under these circumstances, we were prepared to find that histological examination of the bitten areas would produce evidence of a haemorrhage similar to, but larger than, that produced by a feeding mosquito—a supposition which was supported by the appearance of a subcutaneous darkening in all instances after tsetses were fed on thin-skinned laboratory animals, and occasionally after they were fed on man.

THE LESIONS PRODUCED

The above prediction was confirmed by the examination of serial sections cut through pieces of tissue removed from the bitten areas of 20 human and animal subjects, all but one of which revealed the presence of a haemorrhage sufficiently extensive to allow the fly to fill rapidly and completely with blood. If we combine these observations on the lesions produced with those previously recorded on the flexibility of the proboscis, the

method of feeding seems clear. The proboscis is deeply inserted, and then partly withdrawn. When it is again driven into the tissues, the tip takes another path, and so, during repeated insertions, probes the tissues throughout a considerable area, continually injecting the saliva, which is known to contain an anticoagulant (Yorke and Macfie, 1924; Lester and Lloyd, 1928). If, during repeated probings, a blood-vessel is lacerated, a haemorrhage will result. This haemorrhage will spread, and it is from the resulting pool of blood mixed with the anticoagulant that the fly obtains its meal. Since in only one instance did histological examination fail to reveal such a pool of blood, and since we observed the subcutaneous darkening, indicative of the haemorrhage, in all instances when feeding was practised on a further and larger series of thin-skinned animals, we have come to the conclusion that this is the normal, if not the only, method of feeding practised by *Glossina*. There appears little evidence to support the previously accepted view that feeding takes place directly from the lumen of a capillary; and, moreover, it is probable that the majority of the vessels in human skin capable of being reached by the proboscis would be too small to accommodate the labium of *Glossina*.

During the process of cutting the blood-vessels, some of the outpoured salivary secretion will enter directly into the circulation, but the bulk of the fluid will be discharged into the tissues. It follows that the vast majority of the metacyclical forms of *Trypanosoma gambiense* and *T. rhodesiense* introduced by an infected *Glossina* will be deposited in the tissues and distributed over a relatively wide area. This is a different concept from that usually presented in the literature and typified by Lester and Lloyd's (1923) illustration of the proboscis resting in a capillary, with the saliva containing the trypanosomes being discharged directly into the circulation. Under these circumstances, the bulk of any introduced anticoagulant would be immediately swept away, and it is difficult to associate this fact with Lester and Lloyd's statement that sufficient anticoagulant is sucked back to prevent coagulation of blood in the crop.

In view of these findings, we considered it of some importance to determine what further histological changes occurred in the bitten area, since local reactions might affect the subsequent development of any introduced trypanosomes. We therefore cut serial sections of tissues removed from the bitten areas at various times after flies had been allowed to feed. For most of our experiments we employed the technique already described, and fed the flies on anaesthetized guinea-pigs, which were killed either one hour, 24 hours, 48 hours, 60 hours, or 72 hours after the bite; but we also observed the effects of the bites on other animals, and, in order to assure ourselves that the fly fed in a similar manner on man, we examined biopsy material removed from two volunteers within a few minutes of the fly's being fed.

The results of examining this material may be summarized as follows. In cases where the tsetse had been fed on the thigh of a guinea-pig, it was found that the fully extended proboscis could just reach the muscle; its depth of penetration of the forearm of man is probably about the same. It follows that the pool of blood from which the tsetse feeds may be formed anywhere above the muscle layer (Plate XII, fig. 2), but we have observed that some of the uncoagulated blood may sink down between the muscle-fibres, well below the reach of the proboscis (Plate XII, figs. 3 and 4).

At the end of 24 hours the pool of blood is usually extended considerably, not only in the subcutaneous layer (Plate XIII, fig. 5), but also between the muscle-fibres. For up to 48 hours there is no evidence of any disappearance of the red cells, of cellular invasion,

or of degenerative changes, but in from 48 to 60 hours absorption of the haemorrhage takes place, there is evidence of cellular invasion—mainly by histiocytes—and, where the haemorrhage has extended into the muscle layers, there is a slight but distinct degeneration of the immediately adjacent muscle-fibres (Plate XIII, figs. 6–8). At the end of 72 hours the haemorrhage has disappeared and the tissue appears normal. The most outstanding feature in the histological picture as observed in unsensitized animals is the complete absence of any signs of classical inflammation, the appearance and subsequent history of the lesion being such as might be expected to follow the aseptic production of an uncomplicated haemorrhage beneath the skin. Our observations do not suggest that the tsetse injects anything in the nature of a toxic or irritating fluid.

For comparison, we cut serial sections through the skin and underlying tissues of guinea-pigs one hour and 24 hours after the bite of a tsetse-fly from which the salivary glands had been removed by the technique described by Lester and Lloyd (1928). The tissue removed one hour after the bite shows a haemorrhage very similar to that following the normal bite (Plate XIV, fig. 9). After 24 hours this haemorrhage is extended but is greatly reduced in density (Plate XIV, fig. 10), the volume of extravasated blood being considerably less than that in the pool following the bite of a normal fly (see Plate XIII, fig. 5). It is suggested that, when there is no anticoagulant in the surrounding tissue, bleeding from the ruptured vessel ceases relatively soon after the bite; but after the bite of a normal tsetse-fly bleeding continues for a long period, the leakage of blood through the surrounding tissue being correspondingly greater.

The above observations all refer to tissues removed from non-sensitized animals and showing no abnormality other than a haemorrhage in various stages of organization. In order to contrast the lesions obtained in a sensitized subject, passive sensitization was transferred to a rabbit by the intradermal injection of serum from a highly sensitized individual, a tsetse-fly was allowed to feed on an area of skin adjacent to that into which the serum had been injected, and the bitten piece of skin was removed one hour later and sectioned. As a control, the experiment was repeated simultaneously on the same rabbit, with serum from a person only slightly sensitive. For further comparison, a tsetse-fly was allowed to feed on the arm of a highly sensitive individual, the skin and underlying tissues being removed a few minutes after the bite, when the resultant wheal was about 5 mm. in diameter; a control experiment was carried out with an individual who was only very slightly sensitive. In the two experiments with human subjects, a local anaesthetic was used, a subcutaneous injection of 2 per cent. procaine hydrochloride, without adrenaline, being administered just before the tissue was removed.

The lesions in the sensitized human and in the passively sensitized rabbit were similar, and differed from those seen in the controls in that, in addition to the characteristic subcutaneous haemorrhage, there was some dilatation of the vessels and extensive separation of the collagen fibres, to form the wheal associated with the bite (Plate XIV, figs. 11–12). The controls showed little separation of the collagen fibres, and were generally similar to the tissues removed from the (non-sensitized) guinea-pigs.

Thus, in non-sensitized human and animal subjects, the tsetse-bite results in an extensive haemorrhage which may spread into the underlying tissue and persist for several days. The extent of the haemorrhage depends on the injection of anticoagulant by the fly. In sensitized subjects, there is, in addition to this haemorrhage, a swelling caused by separation of the collagen fibres—presumably because of leakage of fluid from the capillaries.

THE REACTION PRODUCED

With one exception, we never observed any clinical reaction to follow the feeding of tsetse-flies on persons not previously exposed to the bites of these insects. The exception was that of an individual who showed a late erythematous reaction, commencing some 18 hours after his first exposure to *Glossina*; but, since in numerous other instances there was no such reaction, it seems probable that this late reaction was caused by some introduced extraneous infection.

Although most references to the reactions following the bites of *Glossina* refer to those associated with the injection of trypanosomes, it is generally accepted that marked reactions to uncomplicated bites, as to those of other insects, are due to sensitization. Lester and Lloyd (1928) have shown that the reaction is caused by some substance in the salivary fluid, and state that it is the saliva which is not sucked back by the feeding fly which causes the wheal at the site of the bite.

In our experience, persons showing a marked reaction to the bites of *Glossina* were always found to have been exposed previously to this fly. During our experiments, tsetse-flies were fed on 18 individuals who had never, to their knowledge, been exposed to *Glossina*, and who, in practically all cases, had never been out of this country. In all but the one case mentioned above no reaction followed the bite. Further tests were carried out on two of these persons, and in each case an immediate reaction was produced by further and irregular feeding. The first subject experienced four bites within two weeks, and on no occasion showed a reaction. Six subsequent bites, experienced simultaneously, gave rise to a typical severe oedematous reaction, which in this instance was not noticed until the following day. Occasional bites during the following nine months each gave rise to a very severe immediate reaction, with wheals as much as 3 cm. across, and to very pronounced swelling of the bitten arm for up to seven days after the exposure. The second individual experienced five single bites at approximately fortnightly intervals. The first three bites produced no reaction, the fourth a slight immediate reaction, and the fifth a severe immediate reaction, which consisted of a large wheal with pronounced swelling and erythema of the surrounding area of the arm. The sensitization reaction is thus far more severe than the immediate reaction following mosquito-bites, and the wheal may occur a considerable time after the bite. In all cases, however, whether delayed or immediate, the reaction consists of a wheal surrounded by extensive erythema, and in highly sensitive individuals is often accompanied by extensive and prolonged swelling of the bitten area.

We did not test the effect of regular feeding on a non-sensitized subject, nor, because of the severity of the reactions, did we test the effect of regular feeding on the sensitized individuals. The evidence of other workers who have fed large numbers of tsetses on themselves suggests, however, that continuous feeding does produce desensitization, and, since the area of the body on which the flies are fed does not react so violently as other areas, that the desensitization is to some extent localized. We have found no evidence that sensitization is at first localized, for, although each of the two individuals who were sensitized by a few bites over a short period received these bites on one arm, they were, when sensitized, equally sensitive on all parts of the body.

The substance responsible for the reaction appears, as in the mosquito, to be present in the saliva. Lester and Lloyd (1928) showed that no reaction followed the bite of a tsetse-fly from which the glands had been removed—an observation which has been

confirmed by our own experiments. An individual who was extremely sensitive to the bites of *Glossina*, normally reacting with a large wheal within half a minute of the bite, was exposed to a treated fly, and although the fly was allowed to probe for five minutes, and became gorged, only an extremely faint reaction occurred, and five minutes after the bite even this had disappeared. Further, we have repeatedly observed that, in sensitized persons, the intensity of the reaction increases with the length of time for which the fly is allowed to feed, thus indicating that the responsible substance is injected throughout the act of biting.

Our observations on the reaction to the bite of the tsetse-fly may be summarized as follows. The only visible reaction is that which follows sensitization; it takes the form of a wheal surrounded by an area of erythema, and, in highly sensitive persons, is associated with severe swelling of the adjacent areas. The substance causing the reaction is contained in the salivary fluid, and is injected throughout the period of biting. This substance is in itself incapable of causing a reaction, and in non-sensitized subjects the only histological lesions are those due to the mechanical trauma caused by the insect's mouth-parts.

DISCUSSION

Our observations on the feeding-mechanisms of mosquitoes and tsetse-flies, and on the lesions and subsequent reactions produced by their bites, have shown that the mosquito may obtain its blood-meal either directly from a capillary or from a haemorrhage in the tissues formed by previous laceration of a capillary (pool feeding); and that, whereas it is probable that the mosquito generally obtains blood by the first method, pool feeding appears to be the normal method adopted by the tsetse-fly. We have also shown that, consequent to the bites of these insects, sensitized persons exhibit histological changes at the site of the bite, characterized by escape of fluid from the capillaries. Following the bites of mosquitoes, but not of tsetse-flies, there may also be a delayed reaction—histologically similar to but not so pronounced as the sensitization reaction—which apparently is caused by the introduction of a slow-acting toxin in the insect's saliva. These changes may be further complicated by the introduction, with the saliva, of such substances as anticoagulins or agglutinins.

It is of some interest to consider the possible effects of these haemorrhages, and of the subsequent tissue changes, on the development of any parasites introduced into the mammalian host. Any parasites introduced directly into a capillary will at once be swept away into the general circulation, but it seems more usual for the feeding insect to deposit the introduced parasites in the tissues, either in or near to the extravasation of the blood. It is these parasites which may be affected by the local conditions, and in view of its possible parasitological significance we made some observations concerning the generality of pool feeding amongst blood-sucking insects.

For this purpose we studied the feeding methods of two insects, *Chrysops* and *Cimex*, which differ widely from each other and from the mosquitoes and tsetse-flies with which we had previously worked, and found that they also invariably adopted the practice of probing the host tissues repeatedly before settling down to feed. This behaviour suggested that they were lacerating the tissues in order to produce a haemorrhage prior to feeding, and subsequent examination of serial sections of tissues removed from the bitten areas confirmed this supposition. Tissues removed one hour after an insect had fed showed

a considerable subcutaneous haemorrhage following the bite of *Chrysops* (Plate XV, fig. 13), and a smaller one following the bite of *Cimex* (Plate XV, fig. 14); while in the tissues removed 24 hours after the bites the haemorrhages were, in both instances, greatly extended.

We investigated the flexibility of the proboscis in *Cimex*, and adopted the same method as that employed with *Glossina*, the proboscis being cut off close to the head while the insect was feeding. Examination of the severed proboscis after the surrounding tissues had been cleared showed that it was slightly curved, and that the mandibles, which were extended beyond the maxillae, were markedly hooked (fig. 8). It is possible that this extension of the mandibles, or retraction of the maxillae, occurred when the proboscis

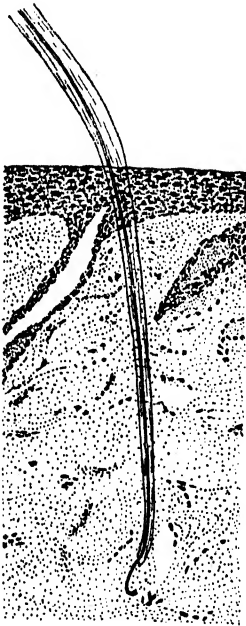


FIG. 8. Diagram, prepared from camera-lucida drawings, of the proboscis of the bed-bug, *Cimex lectularius*, in the act of feeding in guinea-pig skin. (\times about 100.)

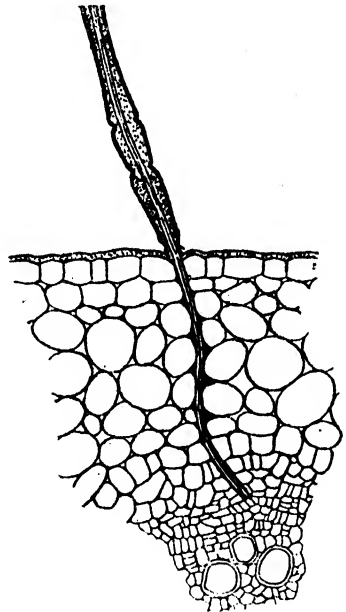


FIG. 9. Drawing of the proboscis of the aphid, *Myzus persicae*, in the act of feeding in plant tissue. (Reproduced, by kind permission, from K. M. Smith's 'Plant Viruses.' Lond.: Methuen.)

was cut, and without further confirmation we have not assumed this to be a normal condition during the penetration of the tissues. It is interesting to note that flexibility of the proboscis has been observed previously (e.g., by Smith, 1935) in the plant-feeding aphids (fig. 9), although in this instance the curvature assumed may be entirely passive.

The employment of a flexible proboscis to lacerate the host tissues over a wide area, and so to produce a haemorrhage from which the insect can feed, is thus characteristic of insects of widely different genera and responsible for the transmission of widely differing parasites. Such a method of feeding will clearly influence the taking up of such parasites by these insects, and—what appears to us of much more importance—will determine the site of their deposition in the mammalian host, for the majority must, in these circum-

stances, be delivered into the tissues or into the blood pool, and not directly into the circulation. It is highly probable that such an environment will condition the development of the parasite—indeed, it may even be essential to the development of certain species.

SUMMARY

1. Flexibility of the proboscis during the act of feeding, similar to that already proved to occur in the mosquito, is shown to occur in *Glossina* and *Cimex*.

2. It is confirmed that the mosquito may feed either directly from a capillary or from a pool of extravasated blood. The second method, referred to as pool feeding, is demonstrated to occur also in *Glossina*, *Chrysops* and *Cimex*.

3. Pool feeding is shown to be the usual method employed by *Glossina* in obtaining its blood-meal, the haemorrhage produced being relatively extensive and large enough for the fly to fill rapidly with blood.

4. The haemorrhage resulting from a tsetse-bite is shown to increase during the first 24 hours after the bite, and in the guinea-pig to persist for up to 48 hours, during which period it may spread into the muscle layers. After 48 hours the area is invaded by histiocytes, and by 72 hours after the bite the tissue is normal. After the bite of a fly from which the salivary glands have been removed, the haemorrhage is less extensive.

5. The immediate reaction to the bite of the mosquito is shown to be due to sensitization. This reaction is produced and maintained by irregular exposure to the bites, but disappears after continued regular exposure. Desensitization of the human host to one genus does not necessarily involve desensitization to another.

6. The delayed reaction to the bite of the mosquito is shown to be caused by the injection of a slow-acting toxic substance in the saliva. This reaction appears after the first exposure, or the first few exposures, to the bite, but disappears after continued regular or irregular exposure.

7. It is suggested that the 'immunity' to mosquito-bites generally exhibited by natives of the tropics is not a racial characteristic, but is due to the fact that from birth they are regularly exposed to the bites of these insects.

8. It is shown that no reaction is experienced by individuals bitten by *Glossina* for the first time, and that all reactions to uncomplicated tsetse-bites are due to sensitization. This sensitization is acquired after as few as five bites.

9. The apparently wide-spread habit of pool feeding by blood-sucking insects is discussed, and it is pointed out that the majority of parasites introduced by insects feeding in this manner will be deposited, not directly into the circulation, but into the tissues in or near to a pool of extravasated blood, and that such an environment may determine their future development.

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FIG. 1. The tip of the proboscis of *Glossina* during the act of penetrating the tissues. The inner walls of the labella are partly everted and the teeth are protruding. ($\times 200$.)

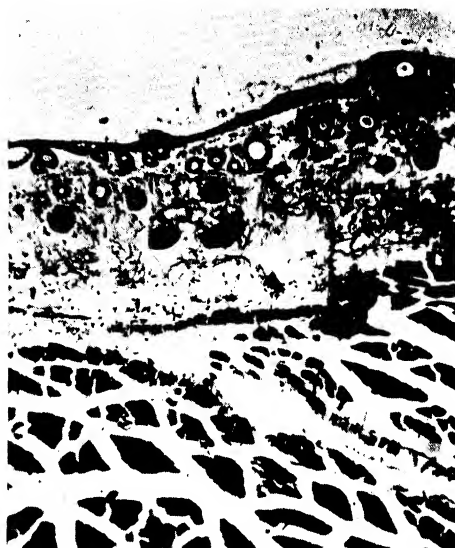


FIG. 2. Section through the skin of a guinea-pig, overlying thigh muscle, one hour after a tsetse had fed on the area. A haemorrhage can be seen lying above the muscle layer. ($\times 65$.)



FIG. 3. Section through the skin of a guinea-pig, overlying thigh muscle, one hour after a tsetse had fed on the area. The originally more superficial haemorrhage has extended deeply between and into the muscles. ($\times 25$.)



FIG. 4. Part of fig. 3, enlarged to show the extensive haemorrhage between the muscle-fibres. ($\times 90$.)

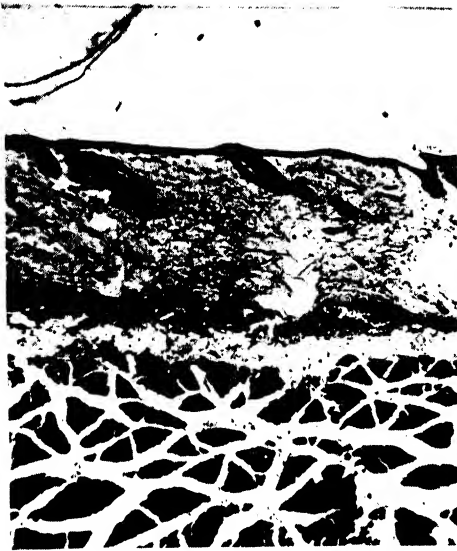


FIG. 5. Section through the skin of a guinea-pig, overlying thigh muscle, 24 hours after a tsetse had fed on the area. The haemorrhage is now deep and very extensive. ($\times 50$.)



FIG. 6. Section through the skin of a guinea-pig, overlying thigh muscle, 48 hours after a tsetse had fed on the area. The haemorrhage is being absorbed, and the area which it occupied is marked by cellular infiltration, mainly by histiocytes. ($\times 25$.)



FIG. 7. Part of fig. 6, enlarged to show the slight degeneration of a few muscle-fibres in the invaded area. ($\times 90$.)



FIG. 8. Section through the skin of a guinea-pig, overlying thigh muscle, 60 hours after a tsetse had fed on the area. The original site of the haemorrhage is still marked by cellular invasion. ($\times 90$.)

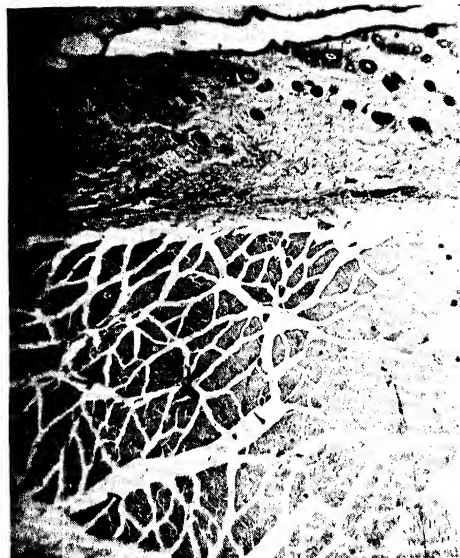


FIG. 9. Section through the skin of a guinea-pig, overlying thigh muscle, one hour after a tsetse, from which the salivary glands had been removed, had fed on the area. A small haemorrhage can be seen lying above the muscle layer. ($\times 25$.)

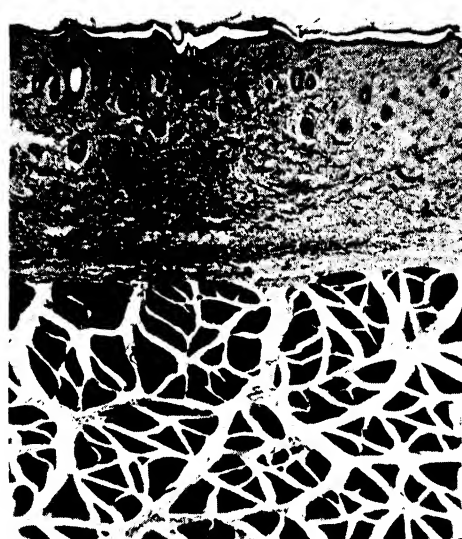


FIG. 10. Section through the skin of a guinea-pig, overlying thigh muscle, 24 hours after a tsetse, from which the salivary glands had been removed, had fed on the area. The haemorrhage is more extended, but is correspondingly reduced in density. ($\times 25$.)



FIG. 11. Section through the skin of the forearm of a highly sensitized individual a few minutes after a tsetse had fed on the area. There is a large subcutaneous haemorrhage and separation of the collagen fibres to form the wheal. ($\times 32$.)



FIG. 12. Section through the skin of the back of a passively sensitized rabbit one hour after a tsetse had fed on the area. There is a subcutaneous haemorrhage and separation of the collagen fibres to form the wheal. ($\times 65$.)



FIG. 13. Section through the skin of a guinea-pig, overlying thigh muscle, one hour after a *Chrysops* had fed on the area. A large haemorrhage can be seen lying above the muscle layer. ($\times 50$.)



FIG. 14. Section through the ear of a guinea-pig one hour after a bed-bug had fed on the area. A small subcutaneous haemorrhage can be seen. ($\times 100$.)

MELARSEN OXIDE IN THE TREATMENT OF HUMAN TRYPANOSOMIASIS

BY

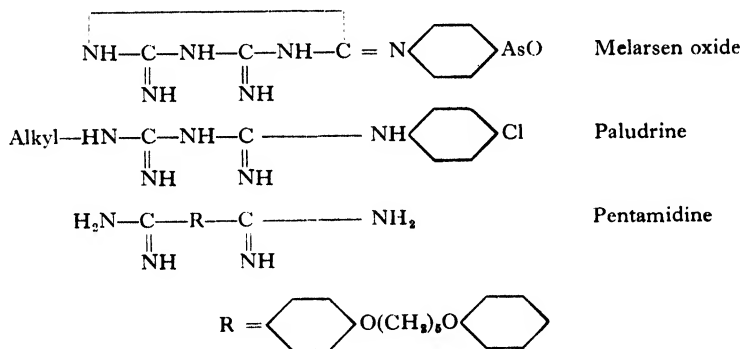
ERNST A. H. FRIEDHEIM*

(From the Laboratory of Ernst A. H. Friedheim, New York, and the Sleeping Sickness Service of French West Africa, Bobo-Dioulasso, Ivory Coast †)

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Melarsen oxide (syn.: *p*-melaminylphenylarsenoxide; *p*-(1:2-diamino-1:3:5-triazinyl-6) phenylarsenoxide) was synthesized and found to combine low toxicity with high trypanocidal activity by Friedheim in 1939.† It is a trivalent derivative of melarsen (syn.: sodium *p*-melaminylphenylarsonate; 'acide triazinearsinique') (see Friedheim, 1940*a*, 1940*b*). Chemically the melarsen compounds differ from the classical phenylarsenic derivatives, atoxyl, tryparsamide, carbarsone, stovarsol, arsphenamine and mapharsen, by their high nitrogen content, expressed by a nitrogen/arsenic ratio of six. For all the tri- and pentavalent arsenicals mentioned above the nitrogen/arsenic ratio equals only one or two.

It is interesting to note that similar structural carbon-nitrogen arrangements occur in the melarsens, in paludrine and in pentamidine:



Results are described below of the treatment of human *gambiense* trypanosomiasis by intravenous injections of melarsen oxide. The field-tests were carried out in two regions of French West Africa, differing in both climatic and epidemiological conditions. The trials were begun in the relatively dry savannah and fly-belt country of the district of Bobo-Dioulasso in the Colony of the High Volta, and were continued in the exceedingly damp forest and mountain region, the home of the Kissi tribe, in the districts of

* In charge of a research mission for the Minister for France Overseas and the High Commissioner for French West Africa.

† Service Général d'Hygiène Mobile et de Prophylaxie.

‡ Chemical and biological data filed with the Bureau Fédéral pour la Propriété Intellectuelle, Berne, Switzerland, December 10th, 1940. See also Friedheim (1944).

Gueckedou and Kissidougou of French Guinea, on the frontiers of Sierra Leone and Liberia.

As a result of the intensive sleeping sickness campaign systematically pursued by the French Sleeping Sickness Service from 1938 through the difficult war-years, the Ivory Coast ceased to be the worst sleeping sickness area in French West Africa, and the incidence of trypanosomiasis was reduced from 2 per cent. (number of persons examined 386,544) in 1939 to a mere 0.25 per cent. (number of persons examined 1,618,455) in 1946.

A number of circumstances have combined to favour a specially high sleeping sickness incidence in the Kissi region of French Guinea. They include ideal breeding-conditions for *Glossina palpalis*; difficulty of access to the population, who inhabit innumerable small villages spread over wide forest and mountain areas, which make systematic surveys very difficult; the independent character of the natives, who submit unwillingly to prolonged treatment and control; and free movement of populations to and from a territory with a high incidence of trypanosomiasis (reaching 26 per cent.; see Veatch, 1946), where no systematic efforts are at present being made to control the disease, and from which infection steadily seeps into British and French territory and counteracts the efforts of the British and French Sleeping Sickness Services. In 1947 the incidence of sleeping sickness in the Gueckedou district of French Guinea reached 8 per cent., despite the fact that in 1946 119,410 persons, out of a population of 175,547, had been surveyed and 1,202 new patients had been detected and treated.

DOSAGE

Melarsen oxide was administered intravenously to 54 cases of sleeping sickness, including 19 cases with pathological cerebrospinal-fluid changes. The preparation was used in the form of a 5 per cent. solution in propylene glycol, dispensed ready for injection in rubber-capped multiple-dose vials. The solution is colourless, clear and slightly viscous.

On the basis of preliminary tests, the dosage for a single injection was set at 1.5 mgm. per kgm. body-weight, without ceiling. The treatment consisted of two series of seven intravenous daily injections, the two series being separated by a rest-period of one month. This dosage was, without exception, well tolerated and caused no gastro-intestinal, circulatory, renal or visual disturbances. Differential blood counts, performed at random on 10 patients, failed to reveal any pathological changes attributable to the treatment.

The intravenous dosage of melarsen oxide in these studies is considerably higher than that used by other authors, as shown below:

Weinman and Franz (1945): seven daily doses of 0.1 mgm. per kgm.

Van Hoof (1947): 15 doses of 0.5 mgm. per kgm. every second day.

Rose and Culbertson (1945): seven daily doses of 0.15–0.2 mgm. per kgm.

THE IMMEDIATE TRYPANOCIDAL EFFECT

All cases were diagnosed by the presence of trypanosomes in glands or blood or both.* In 30 cases cervical lymph-glands, positive before treatment, were re-examined 24 hours after the first injection. In all 30 the glands were cleared of trypanosomes within

* Routine blood examinations were carried out in fresh preparations and in stained thick smears; blood infections were rare, amounting to only two out of 49 cases.

that period. The same trypanocidal effect was found in two cases in which trypanosomes had been found in the blood. During two months following the first injection weekly examination of glands and blood failed in all cases to reveal trypanosomes.

EFFECT ON THE PATHOLOGICAL CHANGES OF THE CEREBROSPINAL FLUID

Observations on 19 cases with advanced cerebrospinal involvement, including 10 with trypanosomes in the cerebrospinal fluid, are summarized in Tables I and III.

TABLE I

Early effects of melarsen oxide on the cerebrospinal fluid in 14 cases of advanced second-stage sleeping sickness. The drug was administered, by intravenous injection, in the form of a 5 per cent. solution in propylene glycol, in doses each of 1.5 mgm. per kgm. body-weight

Treatment	Before treatment			One week after first series			Three weeks after first series			One week after second series			Final result
	Cells	Albu- men	Trypano- somes	Cells	Albu- men	Trypano- somes	Cells	Albu- men	Trypano- somes	Cells	Albu- men	Trypano- somes	
Group I (nine cases, treated with two series of seven daily injections, with a month's interval between the series)	200	0.60	+	5	0.40	0	3	0.35	0	1	0.22	0	Cells normal Albumen normal
	200	0.50	+	3	0.40	0	5	0.38	0	2	0.22	0	
	30	0.40	+	2	0.30	0	4	0.22	0	4	0.25	0	
	32	0.40	+	8	0.30	0	1	0.30	0	1	0.22	0	Cells normal Albumen above normal
	200	0.40	-	2	0.40	0				1	0.35	0	
	80	0.65	0	8	0.56	0				3	0.50	0	
	88	0.45	+	8	0.40	0	2	0.30	0	5	0.56	0	
	340	0.40	++	2	0.40	0	1	0.40	0	2	0.40	0	
	24	0.40	0	1	0.35	0	2	0.25	0	1	0.30	0	
Group II (five cases, treated with seven daily injections)	200	0.50	0	6	0.40	0	1	0.15	0				Cells normal Albumen normal
	160	0.30	+				2	0.18	0	2	0.18	0*	
	96	0.45	0	2	0.40	0							Cells normal Albumen above normal
	23	0.40	0	2	0.30	0	2	0.70	0				
	280	0.65	+	2	0.60	0	2	0.40	0				

* Fifty-seven days after first series of injections

Immediate Effect

Fourteen cases, followed up for one week after the end of treatment, are summarized in Table I, which shows the following results.

Trypanosomes disappeared from the cerebrospinal fluid in nine out of nine cases one week after a single series of seven intravenous daily injections. The cerebrospinal-fluid cell count returned to normal in 13 out of 13 cases within three weeks after the first series of seven injections; in nine out of 13 cases the cell count had become normal after

only one week following the first series of injections. The protein content of the cerebrospinal fluid (total protein determined by the Siccald Cantaloube method) returned to normal as follows :

In one week in 0 out of 13 cases after the first series of seven injections.

In three weeks in four out of 13 cases after the first series of seven injections.

In one week in four out of nine cases after the second series of seven injections.

Clinical improvement followed the drop in the cell count more closely than the improvement in the protein content.

The foregoing observations establish the following facts. (a) Melarsen oxide has a definite trypanocidal action, demonstrable in all localizations, including the cerebrospinal fluid. (b) Improvement in the pathological cell count of the cerebrospinal fluid is rapid, a single series of seven daily injections reducing the count to normal in all 14 cases of this group. (c) The effect on the pathological protein content is slower than the effect on the cell count : one week after the end of treatment only four out of nine cases had returned to normal protein values, though 13 out of 13 cases had returned to a normal cell count.

Discussion of the effects of the drug on the cerebrospinal fluid should not neglect possible irritant actions of the drug, inducing a temporary rise in cell count and protein content. This point, in the case of melarsen oxide, is brought out by an example summarized in Table II.

TABLE II

Summary of the irritant effect of melarsen oxide on the cerebrospinal fluid of a case of first-stage sleeping sickness after treatment with seven daily intravenous injections, each of 1.5 mgm. per kgm. body-weight

Cerebrospinal fluid	Before treatment	After treatment	
		7 days	20 days
Cells per mm. ³	0.80	9	1
Protein, gm. per cent.	0.22	0.40	0.22
Trypanosomes	0	0	0

In practice, only continued observation of the patient can show whether an increased protein content persisting for any length of time after an otherwise successful treatment spells a latent infection or a residual 'scar effect.' These short-term results are obviously not intended to presage any long-range benefit.

Prolonged Effect

Observations on five advanced cases, followed up for 4-10 months after the last injection, are summarized in Table III. Three of these five cases showed trypanosomes in the cerebrospinal fluid. The cases in this group were diagnosed, treated and controlled by the French Sleeping Sickness Service at their headquarters at Bobo-Dioulasso, and the present author is indebted to Colonel Le Rouzic, Director of the Service, for permission to include these data here.

Subsequent to treatment, cell count and protein content of the cerebrospinal fluid reverted to normal in all five cases. In contrast to our observations in French Guinea,

however, the reduction of both cell count and protein content in this group progressed slowly and at about the same rate, taking from three to nine months to reach normal. It is noteworthy that the improvement is progressive and continues for months after the end of treatment. This suggests that the trend of cerebrospinal-fluid changes may be

TABLE III

Late effects of melarsen oxide on the cerebrospinal fluid in five cases of advanced second-stage sleeping sickness. The drug was administered in two series of intravenous injections, each series consisting of seven daily injections of 1.5 mgm. per kgm. body-weight, with a month's interval between the series

Patient	Duration of control*	Glands	Blood	Cells	Protein	Trypanosomes
302/9 Fiekoubo Toure	Before treatment	T+	T+	300	0.45	T+
	2 weeks	TO	TO	39	0.40	TO
	1 month	TO	TO	13	0.40	TO
	2 months	TO	TO	17	0.24	TO
	3 months	TO	TO	3	0.22	TO
	10 months	TO	TO	2	0.22	TO
316/E Sidi Baro	Before treatment	T+	TO	112	0.40	TO
	1 month	TO	TO	18	0.28	TO
	6½ months	TO	TO	2	0.25	TO
416/2 Samory Ouattara	Before treatment	T+	TO	230	0.48	T+
	1 week	OG	TO			
	6 weeks	OG	TO	10	0.35	TO
	2 months	OG	TO	15	0.28	TO
	3 months	OG	TO	12	0.36	TO
	4 months	OG	TO	6	0.22	TO
	5 months	OG	TO	9	0.26	TO
	7 months	OG	TO	4	0.25	TO
	9 months	OG	TO	1	0.20	TO
417/2 Dio Ouattara	Before treatment	T+	TO	152	0.30	TO
	2 weeks	TO	TO	19	0.28	TO
	3 weeks	OG	TO	11	0.22	TO
	2 months	OG	TO	5	0.22	TO
	5 months	OG	TO	4	0.18	TO
	8 months	OG	TO	3	0.18	TO
2888 Gaousso Sanou	Before treatment	T	T	90	0.36	T
	1 day	TO	TO	22	0.40	TO
	1 month	TO	TO	22	0.30	TO
	3 months	OG	TO	3	0.30	TO
	4 months	OG	TO	1	0.22	TO
	10 months	OG	TO	1	0.22	TO

* The time which elapsed between the last injection and the date of lumbar puncture.

T+ = Trypanosomes present. TO = No trypanosomes. OG = Glands disappeared or too small and hard for puncture.

of more importance for prognosis than its absolute values, which leads to the important practical conclusion that it may be unnecessary to rush in with further treatment when the cerebrospinal-fluid picture is still abnormal, if it is tending towards normality.

From the foregoing it follows that melarsen oxide is an efficient remedy in both the

blood-lymph and the meningo-encephalic stages of sleeping sickness, and thus offers the possibility of a treatment for all stages of sleeping sickness with a single drug. This is significant, because at present each of the two stages of the disease calls for treatment with a different drug. A number of drugs, including pentamidine and naphuride (antrypol, moranyl, etc.), have an excellent trypanocidal effect on the blood-lymph infection, but are quite useless in the meningo-encephalic stage. Once the trypanosomes have invaded the central nervous system tryparsamide is the only drug which may save the patient, but tryparsamide is an unsatisfactory trypanocidal agent in respect to the blood-lymph infection.

In view of this, routine mass treatment has to choose between the two following methods of approach.

1. To start with a careful differential diagnosis, based on lumbar-puncture findings. All cases found with normal cerebrospinal fluid (three cells per mm.³ and 0.25 gm. per cent. total protein, according to the standards set by the Brazzaville conference of 1948) may then benefit by the rapid treatment (1-2 weeks) afforded by pentamidine; the lengthy tryparsamide cure (lasting for a minimum of three months) may then be limited to cases with pathological cerebrospinal fluid. (This is the method of the French and Belgian workers.)

2. To obviate the inconveniences of mass lumbar puncture by foregoing a precise diagnosis and by treating all cases as though the central nervous system were involved, i.e., with a combination of drugs including tryparsamide (antrypol-tryparsamide, orsanine-tryparsamide, etc.). (Methods of this kind are in favour in a number of British West African territories.)

The second method has the drawback of extending a long-term treatment, with quantities of tryparsamide, to a large number (at least 40-50 per cent.) of cases where it is basically not indicated, since at least 40 per cent. of all new cases may statistically be expected to be found in the first stage. (The proportion of first- and second-stage cases varies widely from region to region, depending essentially upon the intensity of the local sleeping sickness campaigns. The percentage of second-stage cases detected in 1946 reached 60 per cent. in French West Africa and 10-20 per cent. in the Belgian Congo.)

It is a matter of medical economics and of judgement of local conditions whether or not it is advisable to pander to the natural aversion of the population against lumbar puncture at the cost of additional time and material.

None of the cases discussed in this paper had had any previous treatment. The present observations, therefore, can afford no information on the effect of melarsen oxide on tryparsamide-resistant trypanosomes. No case was encountered which was resistant to melarsen oxide.

It may be recalled that Van Hoof (1947) found that melarsen oxide was active in tryparsamide-resistant clinical cases, a finding recently confirmed by Williamson and Lourie (1948) in laboratory infections in mice.

SUMMARY AND CONCLUSIONS

The therapeutic results obtained in 54 cases of human trypanosomiasis treated with melarsen oxide and observed for a period up to 10 months do not permit generalizations or final evaluation. Nevertheless the following conclusions may be drawn.

1. Melarsen oxide administered intravenously for human trypanosomiasis due to

Trypanosoma gambiense clears the blood and lymph-glands within 24 hours after the first injection.

2. Melarsen oxide eliminates trypanosomes from the cerebrospinal fluid after treatment of only one week's duration.

3. Melarsen oxide may reduce the pathological cell count of the cerebrospinal fluid to normal within three weeks following a first series of seven daily injections.

4. The pathological protein content of the cerebrospinal fluid is usually reduced less rapidly than the cell count, and may take 3-4 months to reach normality.

5. Melarsen oxide enables all stages of sleeping sickness to be effectively treated with a single drug.

6. Within the limits of the dosage indicated melarsen oxide has had no untoward effect—in particular, no harmful action on the optic nerve.

7. Within the limits of the observations so far made, a comparison of the therapeutic effects of melarsen oxide and tryparsamide shows the following points:

(a) Both preparations achieve the same curative effect on the cerebrospinal fluid, i.e., clearance of trypanosomes and return of cell count and total protein content to normal, within substantially the same period of 3-4 months after the commencement of treatment.

(b) The cell count appears to fall more rapidly with melarsen oxide than with tryparsamide, since it may return to normal in two weeks after the beginning of treatment.

(c) Treatment with melarsen oxide in its present form takes one and a half months, i.e., two weeks of daily treatment separated by an interval of one month. This is significantly shorter than the routine tryparsamide cure, which, in the most favourable cases, takes a minimum of seven months, i.e., two series of 12 weekly injections separated by an interval of one month.

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THE RESULTS OF A PRELIMINARY ENTOMOLOGICAL SURVEY OF LOIASIS AT KUMBA, BRITISH CAMEROONS, TOGETHER WITH A DESCRIPTION OF THE BREEDING-PLACES OF THE VECTOR AND SUGGESTIONS FOR FUTURE RESEARCH AND POSSIBLE METHODS OF CONTROL

BY

R. M. GORDON,

L. J. CHWATT*

AND

C. M. JONES†

*(From the Department of Entomology and Parasitology,
Liverpool School of Tropical Medicine)*

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I. INTRODUCTION

The account which follows is based on the results of a preliminary investigation of loiasis at Kumba in the British Cameroons. The investigation was undertaken at the request of the Colonial Medical Research Committee, and was carried out during the months of June and July, 1948; in addition, certain observations made by one of us (L.J.C.) in the same area during June of the previous year are also included. Attention has been concentrated on the entomological aspects of the problem, although certain helminthological aspects have also been considered.

In spite of its undoubted importance, less is known of the epidemiology and control of loiasis than of any other important filarial infection of man. Our knowledge of the helminthological aspects is incomplete, and extends little beyond the morphology of the adult and the appearance and behaviour of the larvae in the peripheral blood of man and their development to the infective stage in the vector, *Chrysops*. At the infective stage the larva is believed capable of penetrating unbroken skin, but beyond that little is known of its further wanderings or development. We believe that both sexes can and do travel extensively in the human host, both before and after they have become mature. This belief is founded on the examination of a limited amount of material obtained at operations and autopsies and on the living worm's occasional appearance at certain sites, particularly when it crosses the eye; such sporadic appearances, however, cannot be taken as representing the full journeyings of the parasite, any more than the occasional glimpse of a person passing before a lighted window can fully account for his movements within the house. Neither are the clinical manifestations of loiasis fully understood, and, although there appears to be little doubt that the persistent and troublesome condition associated with the presence of 'Calabar swellings' is caused by infection with *Loa loa*, and that the swelling and itching are the result of a patient having become sensitized to the adult worms

* Medical Officer in charge of the Malaria Unit, Medical Department, Nigeria.

† Medical Officer attached to the Epidemiological Unit, Medical Department, Nigeria.

or their products, this generally adopted view has never been fully proved. The results of chemotherapeutic studies of loiasis are almost equally unsatisfactory, mainly because no satisfactory method has as yet been found of testing the efficacy of the drugs used by the investigators, since the appearance or disappearance of microfilariae from the circulation can no longer be accepted as a reliable criterion. Thus, it is known that in filariasis in general the disappearance of microfilariae from the peripheral blood following the administration of a particular drug does not necessarily prove that the parent worms have been destroyed, nor does the persistence of the microfilariae after treatment necessarily prove that the adults are still alive.

As regards the entomological aspects of loiasis, although we know that certain species of *Chrysops* are capable of infection with *Loa loa* under laboratory conditions and that they transmit the disease in nature, we are unaware of the life-cycle, oviposition sites, or breeding-places of the two most important vectors in West Africa, *C. silacea* and *C. dimidiata*, and lack all but the barest outline of the habits and ecology of the adult flies.

These considerable gaps in our knowledge concerning the helminthological, clinical and entomological aspects of loiasis suggest that successful control of the disease is unlikely to be achieved without further study, and that much of the work can only be carried out in the tropics, preferably in an intensely infested area. The preliminary survey described in this paper was undertaken with the object of investigating the suitability of the Kumba area as a loiasis research centre, and of estimating what lines of research might most profitably be pursued.

II. TOPOGRAPHY AND GENERAL DESCRIPTION OF KUMBA

Kumba is in the southern Cameroons and is administered under British mandate. The town of Kumba lies about 60 miles north of Victoria, to which it is connected by a motor road. There is a weekly aeroplane service between Victoria, Port Harcourt, Benin and Lagos, so that Kumba is by no means an isolated town, although during the wet season the road joining it to Victoria is sometimes impassable for several days on end.

The population of Kumba consists of some 30 Europeans and an African population of about 6,000. The native village, which lies some 850 ft. above sea-level, between the valleys of the Kumba and Meme Rivers, is spread out in a narrow line, according to German custom, along the Kumba-Victoria roadside. A few Europeans, mainly missionaries, live in or close to the native village, but the majority, together with a few African Government officials, live in bungalows on Kumba Hill, which is in the form of two steep ridges, rising to 1,000 ft. above sea-level, separated by the main road and flanked on either side by thickly overgrown valleys. In addition to these residences, the Government hospital, prison, public works department, and the teachers' training college are all situated on a nearby elevation. It is in these three residential areas that *Chrysops* appears to be most prevalent, and where so many cases of loiasis amongst Europeans have been recorded that Kumba has become the most notorious centre of the disease in West Africa.

The thickly overgrown valleys flanking the residential areas have, at the bottom, densely shaded streams, which for some part of their course run briskly over shallow beds of rock and form pools, often with sandy or rocky bottoms. In parts, however, the course of the streams is impeded by vegetation, the flow of the water is slow, and the bottom is covered by fine sand overlaid with soft mud which is covered with decaying leaves.

It is in such situations that we found, as will be described below, the breeding-places of *Chrysops*. Joining the two main streams in the valleys are numerous smaller streams flowing down from the settlements on Kumba Hill. Some of these are densely shaded and at points present the stagnant conditions described above. In such places we were able to demonstrate the presence of *Chrysops* larvae, but for the most part the streams flowing down the slopes on which the settlements are built are brisk mountain streams with rocky beds, which are unlikely to contain breeding-places for *Chrysops*.

North of the station, at the summit of the hill, Barombi Lake, about a mile in diameter, lies in a volcanic crater with almost vertical walls; so steep is the descent that the German residents have built metal scaling-ladders at the nearest points of approach. It is unlikely that this area forms a source of infection, for, although we did not investigate the northern end of the lake, fly appear to be scarce on the southern end.

III. PAST HISTORY OF LOIASIS AT KUMBA

Kumba is probably one of the most important centres of loiasis in Africa, and, almost without exception, medical reports from 1918 onwards contain references to the density of *Chrysops*, its high infection-rate, and the risk of acquiring the disease by all residents in the area. In addition to these references in the annual reports, filariasis at Kumba has been the subject of special (unpublished) reports by three medical officers, all of whom agree on the risk of infection. The following statements are quoted from these reports. 'Among Europeans Kumba enjoys a most unenviable reputation on account of the ease with which *Loa loa* infection is contracted' (Dr. Davidson, writing in 1945). 'Nearly every European resident in Kumba develops *Loa loa*' (Dr. Bell, writing in 1942). 'Most of the residents of Kumba are affected' (Dr. C. Wilson, quoted by Chwatt, writing in 1947). 'The problem of filariasis in Kumba is of such practical importance and scientific interest that it warrants its detailed investigation by a parasitologist and a medical entomologist stationed at Kumba for at least six months' (Dr. Chwatt, writing in 1947). These statements are supported by the figures for the human infection-rate and the fly infectivity-rate quoted by the writers. Thus Davidson examined 870 Africans in the area, and found 3·7 per cent. of the children and 23·0 per cent. of the adults with microfilariae of *Loa loa* in the peripheral blood, while his dissections of 903 *Chrysops* (mainly *C. silacea*) revealed an infection-rate of 12·4 per cent., 3·3 per cent. of all the flies examined showing the presence of infective forms. Chwatt examined 194 individuals, and found 9·0 per cent. of the children and 16·5 per cent. of the adults infected, while among 32 *C. silacea* dissected two were found to be infected with developing forms of *Loa loa*.

The persistent high morbidity-rate from *Loa loa* amongst Europeans and Africans at Kumba has caused various suggestions for control to be put forward from time to time. Almost all these suggestions were aimed at protection of the individual from the bites of infected flies, and included such proposals as screening of houses, clearing of bush around residences, and the use of repellents. For various reasons, which will be discussed later, none of these suggestions have been widely adopted.

Until 1947 no breeding-places of a proved vector of *Loa loa* had ever been recorded, and, in consequence, no serious attempt at larval control had ever been suggested. In 1947, however, Chwatt (unpublished report) found two pupae of *C. silacea* on the edge of a densely shaded stream in the Kumba area. He suggested the naturalistic control of *Chrysops* by removal of the vegetation along the edges of streams, but drew attention

to the possible danger of an increase in the number of *Anopheles gambiae* breeding-places which might follow the introduction of such a method of control. As an alternative method of larval control, Chwatt advised spraying with DDT in gas oil along the edges of the streams.

IV. THE PRESENT SITUATION REGARDING LOIASIS AT KUMBA AND THE RESULTS OF THE PRELIMINARY SURVEY

During the course of our investigation at Kumba, we were able to confirm the observations of previous workers regarding the prevalence of *Chrysops* and the high incidence of *Loa loa*, both in the fly and in the human population; for these and other reasons, we came to the conclusion that Kumba was particularly well suited as a centre for a filariasis investigation.

Although it is essential that, in any extensive study of loiasis, clinical and parasitological investigations should be pursued conjointly with an entomological survey, nevertheless we believe that the first steps in the investigation should be directed to the determination of the complete life-cycle of the *Chrysops* vectors—particularly as regards the site and nature of the breeding-places—and to the habits, density and infection-rates of the adult flies, since it is on a knowledge of these points that plans for control are most likely to be founded. In view of this suggestion, it may be of interest to record the preliminary results which we obtained at Kumba during one month of the year, as they reveal how much further knowledge of the life-cycle, bionomics and parasitology of *Chrysops* is required before we can confidently recommend any particular form of control which will be generally applicable. On the other hand, our somewhat meagre records do suggest that the control of *Chrysops* in this particular area (Kumba) may not be as difficult as was first believed, and we propose to discuss how best these local methods of control might be further investigated.

1. *Species of Adult Chrysops Observed*

Davidson (1946), during 18 months' residence at Kumba, collected and identified 959 flies, of which 898 were *C. silacea* and 61 *C. dimidiata*. Our results were similar, for, out of a total of 500 flies dissected at Kumba, 480 were *C. silacea* and 20 *C. dimidiata*. Both these species have already been shown to be 'good vectors' of *Loa loa*, and, so far as we are aware, no other species of *Chrysops* have been observed by ourselves or previous workers in the area, although *C. longicornis* and *C. distinctipennis*—the latter species a proved vector in the Sudan—have been recorded from other parts of Nigeria.

2. *The Density and Infective Density of Chrysops*

The period during which *Chrysops* feeds varies considerably with weather conditions, but it seldom commences biting before 8.30 a.m. or continues to do so after 4.30 p.m. The density of *Chrysops* in the station between these hours was estimated by daily posting one collector, armed with a net, in each of nine dwelling-quarters. The nine collectors, who were on duty for equal fixed periods and who were changed round every three days, collected a total of 571 flies in 943 hours, which gives an over-all fly-density of 0.6 flies per boy-hour (F.P.B.). The figures for individual stations varied from 0.3 to 1.0 F.P.B., the highest density being recorded in bungalows closely surrounded by bush. We did not carry out any controlled collecting of flies except at dwellings on the station, and there-

fore we can give no figure for the density of flies in the bush, but it is certainly very much less than in, or in the immediate vicinity of, dwelling-houses; indeed, the capture of flies in the bush, even in close proximity to known breeding-places, was a comparatively rare event.

A total of 500 flies (480 *C. silacea* and 20 *C. dimidiata*) were dissected, and 36 (7·2 per cent.) were found to be infected with filaria, presumably *Loa loa*. Of these flies, 460 were captured in European residences, and 36 (7·8 per cent.) showed infection with microfilariae, infective forms being present in the head or proboscis, or both, of 20 (4·3 per cent.).

3. Relationship between *Chrysops* Infective Density and the Infection-Rate Found in the Human Population

If we accept these figures of fly-density, fly-infection and an eight-hour biting-period as indicative of conditions at Kumba during the months of June and July, i.e., at the height of the *Chrysops* season, then, on an average, each European would be exposed to the risk of infection with *Loa loa* once in every five days. Since our African collectors were inexperienced, however, and a better-trained team would probably have captured a much higher proportion of the flies which alighted to feed, the risk of infection is almost certainly greater than the figure quoted. Such a high infective density at once raises interesting and important questions of whether the infections demonstrated in *Chrysops* were all derived from a human source, and, if so, why an even greater proportion of the European and African population at Kumba do not, at any one time, show evidence of infection with *Loa loa*, as judged by the presence of microfilariae in the peripheral blood.

As regards the first point—whether the infections seen in the flies are all derived from a human source—this can only be settled with certainty by further investigations, such as tracing the source of the blood-meal by precipitin tests, and by a study of the morphology of the larvae found in the naturally infected flies as compared with the morphology of larvae recovered from bred flies infected with known strains of *Loa loa*. For reasons stated later, we consider it probable, however, that the vast majority of the infections found in wild flies were derived from a human source; and, if we accept this view, three other possible explanations might be put forward to explain the disparity between *Chrysops* infective density and the occurrence of loiasis in the human population.

(i) Only a small proportion of the worms deposited by the fly on the human host penetrate the skin and develop to maturity; hence the chances of infection are considerably less than are suggested by the infection-rate in *Chrysops*, and infection is generally not contracted until after a considerable period of residence in the area. This view receives support from a study of the incidence of filariasis in the African population, though, so far as can be determined with the small figures available, not in the European population. At the time of our visit to Kumba there were 35 Europeans living in the area, their periods of residence varying from a few months to 15 years. From replies to a circular letter and from information given by medical officers, it appeared that nine (25 per cent.) of these residents had, at one time or another, shown evidence of infection with *Loa loa*. In this small European population the infection-rate was found to increase with the length of service, though six of the nine Europeans with loiasis had shown evidence of infection within 12 months of their arrival in Kumba, and must therefore have acquired their

infection within a few months of arrival. Further, of the 26 uninfected Europeans, 22 had been in residence in Kumba for less than 12 months.

Amongst the African population, on the other hand, our figures and those of previous observers show that a higher proportion of adults than children are infected, and that infection is seldom recorded in an African child below the age of seven years. Thus, Davidson in 1944 examined a total of 870 Africans at Kumba and found 3.7 per cent. of the children and 23 per cent. of the adults with the microfilariae of *Loa loa* in their blood at the time of examination; while in 1947 one of us (L.J.C.) found 6 per cent. of 110 children and 16.5 per cent. of 84 adults showing microfilariae.

(ii) A much higher proportion of the human population than is indicated by the examination of their peripheral blood becomes infected with *Loa loa*, but the adult worms die out or are destroyed, and thereafter the individual is immune to infection. That this explanation is an unlikely one is shown by our own figures and by the much more extensive figures of Davidson (1946) of the infection-rates in the various age-groups.

TABLE

Showing the proportion of persons in the various age-groups at Kumba with microfilariae of *Loa* in the peripheral blood at the time of examination

Age-group	From Davidson (1946)								From Chwatt (unpublished report, 1947)				
	0-5 years	5-7 years	8-10 years	11-13 years	14-16 years	17-20 years	Adults	Total	0-5 years	5-7 years	8-12 years	Adults	Total
No. examined	20	82	56	60	62	30	560	870	42	39	33	84	198
No. showing <i>Mf. loa</i>	0 (0)	2 (2.4)	4 (7.1)	4 (6.6)	6 (9.6)	5 (16.6)	130 (23.2)	151 (17.3)	0 (0)	5 (12.8)	1 (3.3)	14 (16.0)	20 (10)

Bracketed figures are percentages.

It can be seen from the table that the proportion of persons showing microfilariae in the peripheral blood tends to increase progressively with age; whereas this would not be the case if there were any acquired immunity.

(iii) A high proportion of the worms deposited by the fly on the human host develop to maturity, and the risk of early infection is as great as is indicated by the *Chrysops* infective density; but, although adult worms are present in the host, evidence of their presence is lacking, either because the infection is a unisexual one, or because the sexes have failed to meet, or because, although the sexes have met and microfilariae are being produced, no microfilariae are present at the time when the blood is examined. This third suggestion is supported by previous experiments on animals, and by the frequency with which patients in whom repeated blood examinations have failed to reveal microfilariae nevertheless show evidence of the presence of the adult worms by a previous history of Calabar swellings or of a worm having crossed the eye.

It should be obvious that these and similar problems are of considerable practical importance, and that the assessment of control and curative measures are to a considerable extent dependent on their solution.

4. *Proportion of Male and Female Chrysops*

All the 460 flies collected from the residences on the Kumba settlement were females. In addition, we collected an approximately equal, but unrecorded, number of flies from various sites within or close to the Kumba area, all of which proved also to be females. Presumably, as in other members of the genus, the males of *C. silacea* and *C. dimidiata* are plant-feeders, and therefore do not tend to accompany the females into houses in their search for blood. On the other hand, whereas a search of flowering plants in the neighbourhood of known breeding-places had always yielded a supply of male *Chrysops* in England, similar methods of search in West Africa met with no success, nor was the adoption of other and more elaborate methods more successful. Not only did we extend the search for male *Chrysops* to various other types of vegetation, both near to and remote from breeding-places, which were examined at various hours from dawn until after dusk, but we also tried, without success, to attract males by hanging up, at likely sites, gauze cages containing females. After all these methods had failed, we offered a prize for the first male captured, increasing the reward until it stood at 10 shillings per fly. This is a considerable prize for the local African, and, judging by the number of flies brought to us, it was keenly competed for; but no capture was made, and we are inclined to think that males frequent and probably fertilize the females in the high forest-canopy.*

5. *The Host Preference of Chrysops*

It is probable that *Chrysops* will feed on a wide variety of warm-blooded animals; we have observed it doing so on cattle, as well as on man, under natural conditions, and on monkeys, guinea-pigs, rabbits, white rats and mice under laboratory conditions. In Kumba, where cattle are rare, it is probable that man is its chief host, although a proportion of the flies may obtain their blood from the monkey population which lives in the forest-canopy. Amongst 500 flies collected by us in or near to human habitations, 141 contained blood, which, in all instances where the erythrocytes were still recognizable, was of the mammalian type; but, since no precipitin tests were carried out, we are unable to state its source with greater accuracy.

6. *The Stages of Ovarian Development Observed in Chrysops*

The stage of ovarian development was recorded in each of the 500 female flies dissected. Probably all or most of these flies had been fertilized prior to capture; at any rate, spermatozoa were found in all of the 19 instances in which the spermathecae were crushed and the contents examined. Under the circumstances, we were surprised to find that in no fewer than 492 of the flies the ovaries were completely immature or had reached only a very early stage of development, corresponding to stage two in Christophers's (1911) mosquito classification. It is certain that a proportion of these flies had already oviposited, for 1-6 retained ova were found in nine of the flies with otherwise undeveloped ovaries. The view that a much higher proportion than this of the flies captured in the bungalows had already oviposited is supported by the finding of 'an old blood-meal' in not less than 15 per cent. of the flies, and by the high proportion with infective forms

* Since this was written, Mr. H. Oldroyd has informed us that *Chrysops* has been recorded from the high forest-canopy in East Africa.



Photographs of breeding-places of *Chrysops*. The sites at which larvae were found were usually more densely shaded than is suggested by these illustrations, but such places were too dark for successful photographs to be taken.

The Africans in the lower photograph are searching for larvae with the sieves referred to in the text.

of *Loa loa*, proving that these flies, at any rate, must have fed on a human host not less than 10 days previously.

We have already drawn attention to our failure to collect any males of the two species of *Chrysops* known to be responsible for the transmission of loiasis in the area, and it may prove of some practical importance to discover the habitats of the males and to find out something about the behaviour of the females during the periods when they are not seeking blood in or close to human habitations.

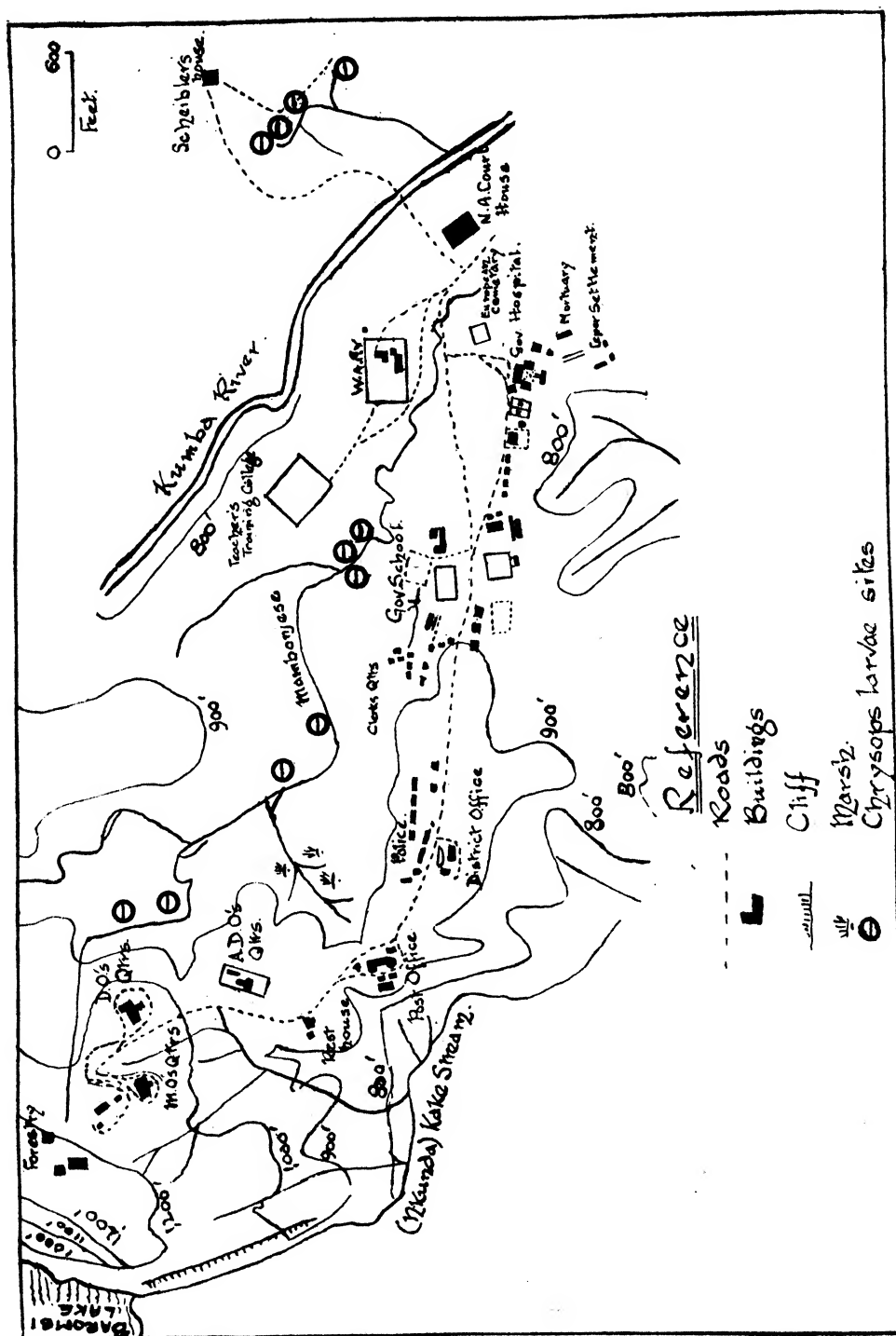
7. *The Breeding-Places of Chrysops*

The difficulty of planning measures to reduce the incidence of loiasis without knowledge of the breeding-places of the vector is obvious; in this connection Brumpt (1936) has written: 'Quand les gîtes larvaires, probablement aquatiques, des deux espèces de *Chrysops*, vecteurs de cette filaire, seront connus, il sera possible, sinon facile, de prendre des mesures prophylactiques.' Since it is proposed that the task of the loiasis investigation team will include the study and application of control-measures against *Chrysops*, much of the time available for the preliminary survey was employed in a search for breeding-places, previously unknown.

In order to train collectors, we had taken with us from England a supply of preserved *Tabanus*, *Haematopota* and *Chrysops* larvae, which we showed to the students at the native schools and at the training college, explaining to them where and how to look for the larvae and offering prizes to the more successful collectors, provided that they could show us the area from which each specimen was obtained. The efforts of these voluntary workers, combined with those of our own staff, produced a regular supply of *Tabanus* and *Haematopota* larvae, and finally of *Chrysops* larvae, the first specimen of which was brought in by an eight-year-old African school-child.

Having established the presence of *Chrysops* larvae in the area, we then settled down to a systematic search of the streams in the neighbourhood, and were able to demonstrate *Chrysops* breeding-places at the various points shown on the attached map; the breeding-places of *Tabanus* and *Haematopota*, which were much more numerous and wide-spread, are not shown. This work of collecting larvae is laborious and slow, but it was rendered much easier and quicker by the use of graduated sieves, mounted in the form of tables, which had been designed by our colleague, Mr. W. Crewe, for his research work in England. We had similar sieves made in Africa, and used them throughout the investigation with complete success.

When we considered that we had made a sufficiently representative collection of preserved specimens of *Chrysops* larvae, we attempted to breed out the remaining specimens under laboratory conditions. Unfortunately, the time left at our disposal was short, and we obtained only one pupa, which failed to hatch, so that we are unable to state with certainty the species of *Chrysops* larvae collected by us. We consider, however, that the majority were almost certainly *C. silacea*, since, with the exception of a few *C. dimidiata*, no other species of adult *Chrysops* was collected in the neighbourhood. Additional evidence in support of this view is supplied by the fact that the solitary pupa bred from a *Chrysops* larva, as well as a single pupa found during the previous survey, corresponded to the two previously obtained in the area by one of us (L.J.C.), which, on dissection, were found to contain *C. silacea* adults. It should be remembered, however, that, although *C. dimidiata* is never as numerous as *C. silacea*, its numbers tend to increase towards the end of the rains.



We have already stated that we consider it probable that many of the *Chrysops* captured by us had previously oviposited ; nevertheless we failed to find any egg masses, nor were we successful in inducing captured females to oviposit in the laboratory.

V. SUGGESTIONS FOR FUTURE CONTROL BY (1) MEASURES DIRECTED AGAINST THE LARVAE AND ADULTS OF *CHRYSOPS*, AND (2) MEASURES DIRECTED TO THE PROTECTION OF THE INDIVIDUAL

1. *Measures Directed Against the Larvae and Adults of Chrysops*

All the European residents at Kumba are familiar with the adult *Chrysops*, locally known as the 'red fly,' and are fully aware of the risk attending its bite ; indeed, there appears to be a consensus of opinion amongst them that the acquiring of loiasis is almost inevitable, and that individual efforts at protection can only postpone the evil and are not worth the inconvenience and trouble involved. Unquestionably this attitude is unfortunate, but in view of the past history of filariasis amongst residents in Kumba, and in the absence of any proved method of successful control, it is not an unreasonable one ; and it appears to us to be unfair to attach unqualified blame either to the individual or to the Government for having previously failed to adopt any particular form of control. This does not imply that we consider the situation at Kumba to be incapable of improvement ; on the contrary, our short survey has convinced us that the risk of loiasis, in so far as the European resident at Kumba is concerned, might be greatly diminished, and possibly eliminated, at a cost relatively insignificant when compared with the damage to prestige and the loss of man-power caused by the present high morbidity-rate.

It is possible that residual spraying with DDT or 'Garamexane' might materially reduce the fly-density, and it is important that this method of control should be investigated ; but we can pass no opinion on its probable value until further experiments in the laboratory and in the field have been completed. On the other hand, our investigation has shown that the breeding-places of *Chrysops* at Kumba are probably very restricted, and that they appear to be confined to certain habitats in densely shaded streams, where slowly moving water passes over a layer of mud covered with decaying vegetation. The streams in and around the European settlement, although densely shaded, run briskly for most of their course over rocky beds, and the task of clearing the bush surrounding the streams and of canalizing the relatively few stagnant portions should not prove a formidable undertaking. If at certain points canalization proves impossible—an unlikely occurrence—larvicides might be employed.

We wish to emphasize, however, that we do not consider that it would be wise to recommend any radical form of control, involving the clearing of the bush round breeding-places, the canalization of streams, or the extensive use of insecticides, until further studies, both in the field and in the laboratory, have greatly augmented our present meagre knowledge concerning not only the effect of such methods on the density of *Chrysops*, but also whether the clearing of bush and the canalization of streams would lead to an increase in the present very limited number of anopheline breeding-places.

2. *Measures Directed to the Protection of the Individual*

Although we believe that the ultimate solution of the loiasis problem at Kumba will be found in larval control, nevertheless our preliminary observations suggest that at least three other methods, directed to protecting the individual from the bites of adult flies,

gave encouraging results, and that, if further investigations confirm their value, they should be employed without delay.

(i) *Screening*. There is a wide-spread objection amongst British residents in West Africa to living in mosquito-proofed houses. Nevertheless, without entering into the pros and cons of the subject, we suggest that, since we have shown the majority of infections to be acquired indoors, the provision of one screened sitting-room in every bungalow would considerably reduce the risk of infection, particularly in the case of women and children. It has recently been shown (Scotland, Department of Health, 1948) that complete protection from midges (*Culicoides*) can be obtained by the use of veils composed of wide-mesh netting previously impregnated with dimethylphthalate (D.M.P.), and it occurred to us that, if such netting offered similar protection against *Chrysops*, it might be employed to screen the doors and windows in houses, without exciting the objections associated with the use of mosquito-netting. In order to test this suggestion we constructed two cages, 4 ft. by 2 ft. by 2 ft. in dimension, consisting of wooden frames with glass at each end and a central sliding frame, which could be covered with unimpregnated and impregnated netting of various meshes. *C. silacea* is markedly positively phototropic, and by alternately pointing first one end of the cage and then the other towards a lighted window we could induce the captive *Chrysops* to pass to and fro through the netting. The results of this experiment were somewhat remarkable, for, although, as we have recorded below, 60 per cent. D.M.P. when applied to the skin gave complete protection, netting soaked in this strength of solution failed to repel the flies, which passed just as readily through the impregnated as through the unimpregnated netting.

(ii) *Repellents*. During our stay at Kumba we found that, though everyone complained of the constant attacks of *Chrysops*, very few made any use of repellents, the general comment being that they 'work against mosquitoes and midges but are no use against *Chrysops*.' In our experience, 'Mylol Cream' containing 30 per cent. D.M.P. gives little or no protection against *Chrysops*, but a single application of 'Liquid Mylol' containing 60 per cent. D.M.P. prevents biting over a period of four hours, the flies alighting but not biting after the first hour. We suggest that this or a similar preparation of D.M.P. should be made available either on payment or as a free issue to all the residents.

(iii) *Clearing the Bush in the Immediate Neighbourhood of Habitations*. Very little clearing of bush around the various buildings has been attempted. This is not due to any neglect on the part of the Government officials, who are most anxious to clear the compounds and only refrain from doing so because the annual grant is not sufficient for the purpose. Periodically, when the money is available, a limited area around one or other of the compounds is cleared, and the residents state that where this has been done there is a decrease in the incidence of *Chrysops*. This opinion agrees with our own findings, in which the highest incidence of *Chrysops* was observed in bungalows lying close to dense bush. We suggest, therefore, that the annual grant should be increased to allow a more generous clearing of bush around the bungalows, and, since flies appear to approach dwellings along even very narrow strips of bush, that such clearings should entirely surround the building.

It should be remembered that, whereas screening and the use of repellents are unlikely to influence the effects of other methods of control, any extensive clearing of the bush around houses, even if remote from breeding-places, would probably do so. It would be essential, therefore, that the effect of such clearing should be estimated as a single measure, i.e., by estimating fly-density before and after clearing, and that other methods of control, such as larval destruction, should not be commenced until this has been completed.

VI. SUGGESTIONS FOR FUTURE RESEARCH

It might be argued that, since we now know the type of breeding-place at Kumba in which *Chrysops* larvae are usually found, it would be advisable at once to institute control-measures directed against the larvae, in order to reduce the incidence of loiasis with the least possible delay. We believe, however, that this would be a short-sighted policy, and we have already explained our reasons for considering that it would be unwise to recommend any radical form of control until we have added considerably to our, at present, only rudimentary knowledge of the life-cycle and habits of the transmitting fly. We consider the Kumba area to be an almost ideal centre for acquiring this much-needed information, and we believe that, when such knowledge has been made available, the task of greatly reducing or even eliminating the risk of infection for persons living in the European residential area at Kumba should not prove impossible, or even very difficult, of achievement.

So far, we have made little reference to the helminthological aspect of the loiasis problem, because, in so short a survey, we considered that our chief attention should be directed to the more pressing and less studied entomological problems. It is true that it will be possible to measure the effects of control-measures directed against *Chrysops* in terms of reduced fly and larval populations, but any attempts to estimate accurately the effect of curative and prophylactic measures on the human population will be dependent on our acquiring considerably more knowledge than we already possess of the life-cycle of the worm and the reactions which it produces in the human host. This is particularly true of serological reactions indicative of the presence of the adult worms, and, until such knowledge has been acquired, chemotherapeutic investigations must of necessity be considerably hampered.

We record below some of the more obvious lines of investigation along which we think future research might be directed. We would suggest, however, that any preliminary experiments in larval control or in chemotherapy which are likely to affect fly-density or fly infection-rate should be carried out, not in Kumba itself, but in some of the small villages around Kumba, where it would be possible to test a particular method of control without affecting neighbouring villages, which would be left free for other experiments. When the value of these tentative experiments has been assessed, the attempt to control loiasis in the residential area at Kumba should be put into operation, using one or more of those methods which have proved most satisfactory in the preliminary trials.

The lines suggested for future entomological and helminthological investigations are as follows.

Entomological. (i) Collection, identification and dissection of tabanids and other possible vectors in the area. (ii) An all-the-year-round survey of the adult *Chrysops*-density and infection-rate. (iii) An all-the-year-round survey of *Chrysops* oviposition, larval and pupal sites. (iv) A study of the complete life-cycle of *Chrysops* under laboratory conditions, and the effect of alterations in temperature, light-intensity, etc., on the various stages. (v) Food requirements of the adult female flies, with particular reference to host preferences as determined by precipitin tests. (vi) Food requirements of the larvae, and their possible association with the limited distribution of the breeding-places at Kumba (so far as we are aware, the food requirements of *Chrysops* larvae are unknown, although it has been suggested that, unlike those of *Tabanus* and *Haematopota*, the larvae of *Chrysops* are saprophagous). (vii) Studies of various methods of control, including the use of

naturalistic methods and insecticides, at all stages of development, and, in the case of adult females, the value of repellents. (viii) The habits of male and female adult flies, with particular reference to resting-places, hours of activity, range of flight, mating habits, tropisms, etc. (ix) The effects on the breeding-places of mosquitoes, particularly of anopheline mosquitoes, of methods directed against the adult and larval stages of *Chrysops*. Such an investigation would be greatly assisted by surveys of the plant- and tree-life carried out before and after clearing.

Helminthological. (i) Susceptibility of *Chrysops* to infection with the microfilariae of *Loa loa* taken up from the human host; their effect on the fly, and the influence, if any, of previous ageing of the microfilariae on their development in *Chrysops*. (ii) The identity of the larvae found in naturally infected flies. (iii) A study of the life-cycle of *Loa loa* in, at any rate, its early and its final stages: (a) the numbers of infective larvae deposited on the skin by *Chrysops*; (b) whether they are deposited only when the fly is actually biting; (c) the proportion of deposited larvae which penetrate the skin; (d) the susceptibility of the larvae to environmental conditions, such as drying, and to insecticides and repellents previously applied to the skin; (e) whether the larvae which penetrate undergo any development at the site of penetration; (f) the remaining stages of development and any lesions produced by them, as studied in material obtained at operations and autopsies, or possibly by the inoculation of susceptible animals. (iv) The preparation of a suitable antigen for assessing *Loa loa* infection; previous observations do not appear to have been carried out with a sufficiently specific antigen, and it is suggested that adult *Loa loa* might be obtained for this purpose at operations or at autopsies performed on the human population, while it is also possible that a sufficiently specific antigen might be obtained from worms belonging to the same genus and occurring in monkeys in the area. (v) A survey of the incidence of loiasis in the population, with special reference to age, sex, occupation, length of residence, etc., as assessed by microfilariae in the circulation, by Calabar swellings, by history of the appearance of the worm in the eye, and, if a suitable antigen becomes available, by serological reactions. (vi) A survey of possible monkey and other reservoirs of loiasis.

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OBSERVATIONS ON *LITOMOSOIDES CARINII* (TRAVASSOS, 1919) CHANDLER, 1931

I.—THE DEVELOPMENT OF THE FIRST-STAGE LARVA

BY

W. E. KERSHAW

(From the Department of Entomology and Parasitology,
Liverpool School of Tropical Medicine)

WITH A STATISTICAL ANALYSIS BY

R. L. PLACKETT

(From the Department of Applied Mathematics, University of Liverpool)

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INTRODUCTION

Most of the recent advances in the treatment of filariasis have followed the adoption by Culbertson and Rose in 1944 of the cotton rat naturally infected with *Litomosoides carinii* as an experimental infection for preliminary chemotherapy in animals. Previously, for lack of a better infection, dogs harbouring *Dirofilaria immitis* had been employed; but, though it was easy to procure naturally infected hosts, transference of infection, although simple, was by no means certain, and the incubation-period was long; furthermore, the results of drug therapy could not be applied directly to filariasis in man because of the difference in the tolerance of the two hosts to some of the drugs employed. Efforts were therefore made to find other more suitable strains. Filariae occurring in imported birds and reptiles were investigated, but satisfactory vectors for the former could not be found in this country, and cold-blooded animals were unsatisfactory subjects for chemotherapy. Cotton rats, however, are easy to breed and to rear, and can be maintained with ease in the laboratory, as can the arthropod vector. The experimental transfer of infection to an uninfected animal can be controlled and its subsequent development predicted with some certainty (Bertram, personal communication, 1948). The adult worms are easily accessible, and all can be identified with certainty, as they lie free—at least in the early stages of an infection—in the serous sacs of the thorax, while the development and evolution of the infection is rapid and is convenient for the assay of drugs.

Some of the stages of the life-cycle are already known in detail. The adult female mite, after having fed on an animal whose blood contains microfilariae, becomes infective in about 15 days. The microfilariae may then be transferred at the next blood-meal, though the exact manner in which this is done is not known. Immature worms no longer than infective forms, though anatomically distinct, have been found in the pleural cavities (Cross and Scott, 1947), though the length of time taken to reach the pleura is not stated. The final moult to the adult form has been found to occur in both sexes when they are 7 mm. long. When the females are half grown the males are nearly fully grown; the average size of the female when fully grown is about 7 cm. and that of the male about 2 cm., these lengths being reached in some cases 10 weeks after infection (Scott, 1946).

The microfilariae are commonly found in the peripheral circulation on, or shortly after, the 50th day of infection. They then become more numerous during the next three months, at the end of which period they reach a more or less steady level, at which they remain for some six months or longer; after that the count gradually falls until the blood finally becomes free of microfilariae, the infection having undergone a spontaneous cure. An animal infected in its youth may thus outlive its parasite, and it seems preferable, therefore, to treat a rising or stable infection induced in the laboratory rather than one which already may be undergoing spontaneous remission in a cotton rat infected in the field (Kershaw and Bertram, 1948).

Of the drugs which have been found to be effective, some are assumed to be active against the adult worm, causing at least sterility, others to be lethal to the microfilariae already in the peripheral circulation and to have less effect on the adults. Histological changes in the uterus of the female, the death of the worms and their encapsulation in reactive fibrosis, and the disappearance of the microfilariae from the circulation are interpreted as criteria for proving the killing of the adults in the pleura, while the disappearance of the microfilariae without pronounced resorptive changes round the adults is taken as indicating effect solely on the microfilariae. It has been suggested elsewhere (Kershaw and Bertram, 1948) that these criteria are by no means clear-cut, for the fall in the microfilarial count may occur naturally at the end of the infection, while the reaction surrounding the adults may arise in untreated cases within three months of infection. Moreover, if the disappearance of the microfilariae is to be used as an index of the death of the parent or of a sudden change in uterine function, it seems essential to know the length of time for which they persist in the peripheral circulation subsequent to the death of the parent, and the time which they take after their release in the pleura to pursue their migration to the peripheral blood.

Parasitic nematodes usually undergo one or more moults during their migration in the final host, and usually increase in length and complexity. In investigating the migration-time of the microfilariae of *L. carinii* it is of advantage to define the early larval stages and to describe their morphological development, for subsequent correlation with the results of direct experiments. It is the purpose of the present paper to record some observations on the early morphological changes; in a further paper we propose to correlate them with the fairly rapid migration-rate.

MATERIAL AND METHODS

It was at first hoped to find some significant and constant morphological feature by means of which it would be possible to distinguish between the microfilariae which had just issued from the vulva, those which were lying free in the pleural cavity, and those appearing later in the peripheral blood. But from preliminary experiments it became clear that, apart from the shedding of the vitelline membrane, no striking constant change occurred during this part of the life-cycle, for though the nuclei usually became progressively more complex in character and arrangement, and though the larvae generally increased in length, it was not possible to measure these changes when a small number of larvae were compared, as the variation in a small sample taken from any one of three sites was too wide to demonstrate an obvious difference. Accordingly it was decided to measure the length of a sufficiently large number of microfilariae from each source to render a statistical interpretation of the results possible, and, though a similar statistical

comparison of the increase in nuclear complexity and arrangement could not be made, these gradual changes can be described and correlated with the changes in length.

It is not easy to estimate the length of the microfilariae. The adult nematode is not a rigid structure, but depends for its size and shape on the interplay of the somatic musculature upon a soft and elastic cuticle; in the larval stage, when the organism is little more than membrane enclosing undifferentiated nuclei, the change in shape induced by alteration in the environment may be extreme. Lapage (1937) showed that the distortion produced by variations in the salt content of the environment can be so extreme that the free-living larvae of the intestinal nematodes, and even their first parasitic stages, can lose all resemblance to nematodes; yet recovery after such a violent change is rapid when the right osmotic conditions are restored. Fülleborn (1929) observed that the length of the microfilariae of the same species at the same time in the blood may differ by 20 per cent., and that some species, in particular *Microfilaria ozzardi*, are capable, whilst alive, of shrinking and lengthening independently of external stimuli. It is necessary, therefore, to subject these very changeable organisms to standard conditions which can be reproduced easily and directly before each experimental measurement. Fülleborn (1929) investigated the alterations produced in the same species of microfilariae by different methods of collection and fixation, and found that a thick film made from the saline suspension after the blood had been centrifuged with normal saline, a moist film fixed in 70 per cent. alcohol, and a simple dry thick film all gave results which did not differ widely from those obtained by the photostatic measurement of living organisms in the blood. He finally recommended that the blood be mixed with 5 per cent. formalin, and, as the microfilariae undergo continuous shrinkage for some days, that they should be examined after a fixed interval—say, one day. However, this method could not be applied to the microfilariae found in the pleural cavities of cotton rats, as it is unusual to find sufficient exudate to allow the microfilariae to be withdrawn directly without the interference of first washing with saline or serum.

A further difficulty was encountered in the variation in length between those at the centre of a film and those at the periphery, where the rates of drying in a thick film differ markedly. This variation is very wide indeed in a thick film of the peripheral blood containing the microfilariae of *L. carinii*, which is allowed to dry and then dehaemoglobinized with water or isotonic saline, and stained with Mayer's haemalum. This difference can be seen without measurement, for those at the centre are compact, short and intact, whilst those at the edges are long and swollen, with the line of nuclei broken into fragments.

In consequence, in order to exclude these fallacies, we used a method of instantaneous fixation, in which a thin film, still wet, was placed in Zenker's fluid, modified by the addition of 5 per cent. formalin, at a temperature of 70° C. In making the films both from the thorax and from the peripheral blood one second or so was allowed to elapse between the spreading of the film and its immersion in fixative.

The films were made from animals infected experimentally in the laboratory, which had a high count of microfilariae in the peripheral blood, and which were in an established stable phase of infection, so as to ensure that the adults were still in active production. All had been exposed to infective mites for one or two days only, so that all the female worms in the rats should be of the same age. The films of the peripheral blood were made from an incision at the tip of the tail before the animal was anaesthetized by the intraperitoneal

injection of 0.02-0.04 c.cm. of nembutal, but in one case (cotton rat 147) further films were made from a fresh incision in the tail after the anaesthetic had been given. The abdomen was opened and the animal was then bled quickly by aspiration from the inferior vena cava, in order to prevent the pleura from subsequently becoming contaminated with blood containing microfilariae which had undergone migration.

Within a quarter or half minute of the abdominal section the thorax was opened with scissors, by two lateral incisions placed as closely to the mid axillary line as possible. The sternum and the attached pectoral muscles were then reflected over the head, thus leaving the mediastinum and the collapsed lungs easily accessible. Films were then made either by passing the slides across the viscera which projected above the cut margins of the thorax, or by taking a group of worms on the end of a glass rod pulled out into a fine round hook, and pulling them across the slide. In one of the cotton rats (no. 152) sufficient exudate was present in the pleura for additional films to be made from the fluid after aspiration in a warm Pasteur pipette.

After fixation, the films were washed overnight with water and passed through alcoholic iodine and sodium thiosulphate solution before staining with Weigert's haematoxylin. It was found that the most certain definition and the greatest uniformity was produced by deeply overstaining the nuclei by immersion in the stain for half an hour, followed by a quick rinse in acid alcohol before washing for an hour. The nuclei of the head and tail were thus sharply defined in all the specimens, though the staining of the rest of the structure might vary. The microfilariae were then measured from the anterior end of the first nucleus to the posterior end of the last.

The measurement was made by means of a Zeiss camera lucida, by which the image was projected on an adjustable table. Each microfilaria was drawn, the nuclei were defined, and the length was compared by a map-measuring wheel against the projection of a stage micrometer made by the same apparatus. Care was taken to ensure that the distances subtended on the table were the same for each part of the microscopic field. Each slide was examined under oil immersion with a no. 4 eyepiece so that the microfilariae occupied about half the field, and the slide was moved along a longitudinal traverse as the straightened microfilariae lay in that direction. There was thus little difficulty in deciding whether an organism should be included or rejected. The microfilariae cutting the field were moved to the centre and returned to their original positions before moving on, thus preserving the line of advance. Only those microfilariae which were entirely in the field or which cut the field below were measured on each traverse, and on the completion of one row the field was moved up one field's diameter. By this means every microfilaria in the slide was counted once, and once only.

RESULTS

It was found possible to study the microfilariae of *Litomosoides carinii* at three stages in their development: firstly, those which have just issued from the parent female and were still lying in her immediate neighbourhood on the mediastinum; secondly, those which had moved to the general spaces of the pleura; and, finally, those which had migrated to the peripheral blood-stream. The larvae observed in the films made from the mediastinum could be separated into two groups: those at the earlier stage of their development, which were present in smears obtained by passing the adult worms across the slide, or by passing the slide across the mediastinum to which adult worms had previously adhered



FIG. 1

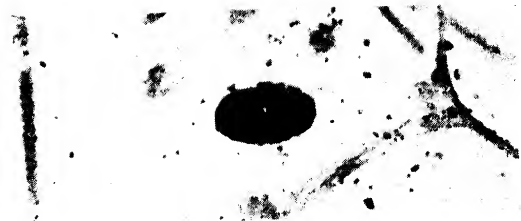


FIG. 2

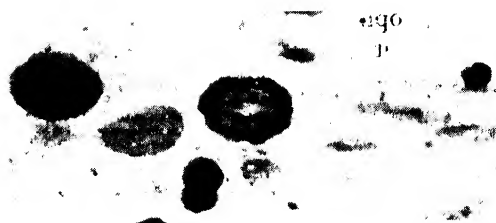


FIG. 3



FIG. 4



FIG. 5

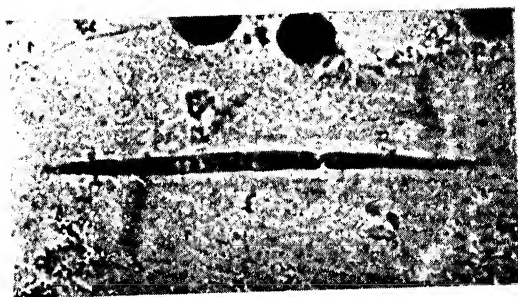


FIG. 6



FIG. 7

and which contained exudate from the immediate vicinity of the adult ; and a slightly less primitive type of larva, which predominated in smears made from exudate lying on the mediastinal surfaces at sites remote from the adults, and which were indistinguishable from those larvae found in the free fluid lying in the pleural sac of one of the rats.

Developing Forms Found in Close Proximity to the Adult Female

In the films made from the fluid in contact with the adult worms a very few eggs, about 15μ in diameter, were sometimes present near the vulva ; eggs in the earliest stage of development contained about 10–15 nuclei with no obvious organization (Plate XVII, fig. 2), but in the more mature ova the nuclei were arranged in a coiled pattern suggestive of a developing larva (Plate XVII, fig. 3). Eggs at a later stage of development were more commonly seen ; these contained an undeveloped larva folded upon itself and still enclosed in its vitelline membrane. The next stage of development, which was the commonest form observed near the adult, was the fully formed and extended larva, which had by now shed its vitelline membrane. These larvae contained 30 or 40 small well-defined nuclei, arranged in no constant pattern and well separated from each other and from the cuticle by clear spaces. In these primitive larval forms there was no suggestion of the nuclear arrangement seen later in the mature microfilaria, nor were the excretory vesicle or 'G' cells present at this stage of development (Plate XVII, fig. 4). Aberrant or deformed larvae not conforming to any of the forms described were sometimes, but very rarely, encountered. The average length of the larvae near the worms was about 65μ , though there was a wide range of variation, for very short forms were not uncommon and, as might be expected, there were small differences between the length of the progeny of individual worms (Table V and fig. 5).

Since the presence or absence of the vitelline membrane or the sheath forms an important guide to the stage of development, they were studied with some care. The vitelline membrane could not be seen as a separate structure round the morulae in the eggs, possibly because it had been fully stretched by the contents ; and, though it was very clear round the primitive coiled larvae, it could not be seen round those which were further developed and which had become extended, presumably because it had been shed. Indeed, empty vitelline membranes varying in shape from spheres to long sheaths were very common, and near the vulva, where most of the morulae and primitive larvae were to be seen, the membranes vastly outnumbered the larval forms (Plate XVII, fig. 1). Some of the membranes were undergoing phagocytosis by endothelial cells and giant-cells, and some

PLATE XVII *LITOMOSOIDES CARINII* DEVELOPMENT OF THE FIRST-STAGE LARVA

FIG. 1. A film of the fluid near the vulva of an adult female, showing early larvae, most of which have shed their vitelline membrane, and many empty membranes.

FIGS. 2–5. Developing forms found near an adult female. Fig. 2 : an undifferentiated morula. Fig. 3 : a morula with a linear arrangement of the nuclei. Fig. 4 : a formed larva enclosed in the vitelline membrane. Fig. 5 : an extended larva which has shed its membrane ; this form is the one most commonly encountered at this site.

FIG. 6. A developing form in the pleura : an unsheathed larva showing for the first time gaps in the nuclear column.

FIG. 7. A form in the peripheral blood : a fully developed larva with a sheath. The excretory vesicle can be seen overlapped by adjacent nuclei.

All sections were fixed in hot formal-Zenker's solution and stained with Weigert's iron haematoxylin.

Scale of figs. 2–7 : 4 cm. = 50μ .

contained basophilic debris, which may have been left behind by a hatched larva or may have been the remains of a degenerate embryo.

Developing Forms Found in the Pleural Cavity

The films made from the fluid in the pleural cavity and those made from the mediastinum which had not been in contact with the adult worms contained more advanced larval forms, and the primitive types seen near the adults were correspondingly rare. The nuclei of these more advanced larvae were larger and more numerous, and had encroached upon the surrounding clear space, often overlapping each other, so that despite their size they were more difficult to define. Whereas no constant arrangement of the nuclei could be seen at the head, the last three or four nuclei at the tail were usually cylindrical

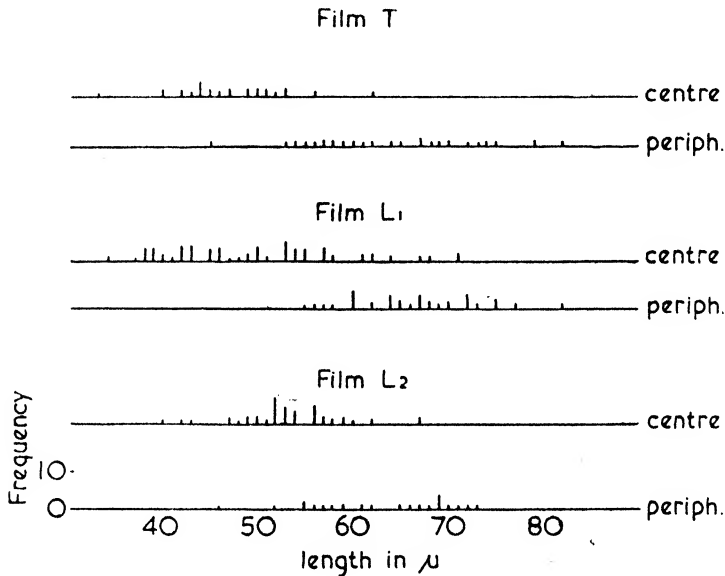


FIG. 1. Frequency-distribution curves of the lengths of larvae of *L. carinii* at the centre and at the periphery of three thick blood films taken from a rat's tail (T) and leg veins (L_1 , L_2). The films were allowed to dry, and were then dehaemoglobinized and stained by Mayer's haemalum. Note the marked difference in length of larvae at the centre and those at the periphery.

and were separated from the rest of the body by a small gap in the nuclear column. At this stage the excretory vesicle and cell could not be stained by Unna-Pappenheim stain, although a tendency to separation in the nuclear column suggested their presence.

The average length of the larvae found in the pleura was about 5 or 6 μ longer than those forms found in the immediate vicinity of the adult worms, and, although shorter forms were present, they were relatively uncommon.

Apparently all the larvae, by the time they reached the pleura, had shed their vitelline membranes—at any rate, no empty membranes, such as commonly occurred in the material obtained from the vicinity of the adults, were seen in the pleura. It would appear that, at this stage of development, the larva for the first time might develop a separate sheath; but the number which do so must be very small, for it was seen only on very few occasions.

Forms Found in the Peripheral Blood

The larva found in the blood were much more uniform both in size and in nuclear arrangement than those recovered from the pleura or found near the adult worms, and there were clear signs of further development. The nuclei were larger and more numerous, almost filling the cuticle, and their arrangement was more orderly. In the centre of the larvae the nuclei were often superimposed, producing an appearance not unlike that of a ship's banded camouflage, through which the vesicle could sometimes be seen as a gap

TABLE I (a)

Comparison of the measurements of larvae at the centre and at the periphery of a thick blood film allowed to dry, dehaemoglobinized and stained. (Cotton rat 132, October 13th, 1947; blood taken from the tail, T)

Larvae found at centre		Larvae found at periphery	
Length, in μ	No.	Length, in μ	No.
33	1	45	1
40	2	53	1
42	2	54	1
43	1	55	1
44	3	56	1
45	2	57	2
46	1	58	1
47	2	59	1
49	2	60	1
50	2	61	1
51	2	62	1
52	1	64	1
53	2	65	1
56	1	67	2
62	1	68	1
		69	1
		70	1
		72	1
		73	1
		74	1
		75	1
		79	1
		82	1
Mean length ...	47.12 μ		64.08 μ
No. of observations	25		25
Sum sq. from mean	856.64 μ^2		1,925.84 μ^2
Estimated variance of mean ...	1.428 μ^2		3.210 μ^2
Difference of means		= 64.08 - 47.12 = 16.96 μ	
Estimated variance of difference		= 1.428 + 3.210 = 4.638 μ^2	
Estimated standard error of difference		= 2.154 μ	

in the column of nuclei, though it might be obscured by the surrounding chromatin. The 'G' cells and the excretory cell could now, for the first time, be stained with Unna-Pappenheim stain with some certainty. The tail nuclei were quite clear; the terminal two or three were usually longer than the others, and were often separated from the rest of the body by a gap. The nuclear arrangement was thus quite unlike that of any of the microfilariae found in man, where the nuclei are usually small, discrete and distinct, and where much of the structure is of cytoplasm. A sheath could be found round some of

the larvae in the peripheral blood, though round others its presence could not be demonstrated.

The slight, but statistically significant, increase in length of the larvae migrating from the mediastinum to the pleura is continued in the journey to the blood, the blood forms measuring about 5μ longer than the pleural forms and about 10μ longer than the

TABLE I (b)

Comparison of the measurements of larvae at the centre and at the periphery of a thick blood film allowed to dry, dehaemoglobinized and stained. (Cotton rat 132, October 13th, 1947; blood taken from a leg vein, L_1)

Larvae found at centre		Larvae found at periphery	
Length, in μ	No.	Length, in μ	No.
34	1	55	1
37	1	56	1
38	3	57	1
39	3	58	1
40	2	60	5
41	1	62	2
42	4	64	3
43	4	65	2
45	3	66	1
46	3	67	3
47	1	68	2
48	1	69	1
49	2	70	2
50	3	72	3
51	1	73	1
53	5	75	2
54	3	77	1
55	3	82	1
57	3		
58	1		
61	2		
62	2		
64	1		
67	1		
68	1		
71	2		
Mean length ...	49.79 μ		66.12 μ
No. of observations	57		33
Sum sq. from mean	4,689.47 μ^2		1,339.51 μ^2
Estimated variance of mean ...	1.469 μ^2		1.268 μ^2

$$\begin{aligned}
 \text{Difference of means} &= 66.12 - 49.79 = 16.33\mu \\
 \text{Estimated variance of difference} &= 1.469 + 1.268 = 2.737\mu^2 \\
 \text{Estimated standard error of difference} &= 1.655\mu
 \end{aligned}$$

mediastinal forms (Table V and fig. 5). Once the peripheral blood is reached growth apparently reaches its maximum; at any rate, the size of the blood forms is much more uniform than those in the mediastinum and the pleura.

In establishing the relatively small increases in length occurring with development it was necessary to eliminate any alterations produced by factors which had not been controlled. The difference in length of the microfilariae at the centre and at the periphery

of thick films has already been mentioned and is shown in Table I (a), (b) and (c) and in fig. 1. A similar analysis of a thin film, treated as described, showed a much smaller difference in the two zones of the film, and a reasonable frequency-distribution curve was obtained from 500 microfilariae (Table II and fig. 2), any difference between the two populations being insufficient to interfere with the results. Some of the blood films were obtained before an anaesthetic had been given, and, as those from the worms and the pleura were made after nembutal had been administered, it became necessary to exclude the possibility of any change in length being due to the effect of nembutal upon the larvae.

TABLE I (c)

Comparison of the measurements of larvae at the centre and at the periphery of a thick blood film allowed to dry, dehaemoglobinized and stained. (Cotton rat 132, October 13th, 1947; blood taken from a leg vein, L_2)

Larvae found at centre		Larvae found at periphery	
Length, in μ	No.	Length, in μ	No.
40	1	46	1
42	1	52	1
43	1	55	2
47	2	56	1
48	1	57	1
49	2	58	1
50	2	59	1
51	1	61	1
52	7	62	1
53	4	65	1
54	3	66	1
56	5	67	1
57	2	68	1
58	1	69	3
59	2	70	1
60	1	71	1
62	1	72	1
67	2	74	1
Mean length ...	53.33 μ		62.91 μ
No. of observations	39		21
Sum sq. from mean	1,242.67 μ^2		1,145.81 μ^2
Estimated variance of mean ...	0.839 μ^2		2.728 μ^2

$$\begin{aligned}
 \text{Difference of means} &= 62.91 - 53.33 = 9.58\mu \\
 \text{Estimated variance of difference} &= 0.839 + 2.728 = 3.567\mu^2 \\
 \text{Estimated standard error of difference} &= 1.888\mu
 \end{aligned}$$

However, in Table IV and fig. 4 it can be seen that the larvae in the peripheral blood were slightly longer after nembutal had been given, thereby implying that the difference between the larvae from the pleura and from the blood would have been greater had it been possible to avoid the use of the drug.

It would appear to be convenient at this point to summarize briefly the development of the larva of *L. carinii* from its birth to its appearance in the peripheral circulation. The embryo may be found near the vulva of the adult female as an egg in one of several stages of development: as an undifferentiated morula, or as a morula in which the cell mass

shows early organization suggestive of a developing larva ; as an egg containing a primitive larva bent upon itself but still enclosed in its vitelline membrane ; or possibly as an extended larva. The majority of the extended larvae near the parent worm have shed the vitelline membrane, as is evidenced by the vast numbers of empty membranes found near the adult female ; in any case, the membrane is always cast before the larva reaches the general spaces of the pleura. On reaching the pleural spaces the simple larva has developed further to a form in which some features suggestive of those found later in the mature microfilaria can be seen ; but at this stage a separate larval sheath, as distinct from the early

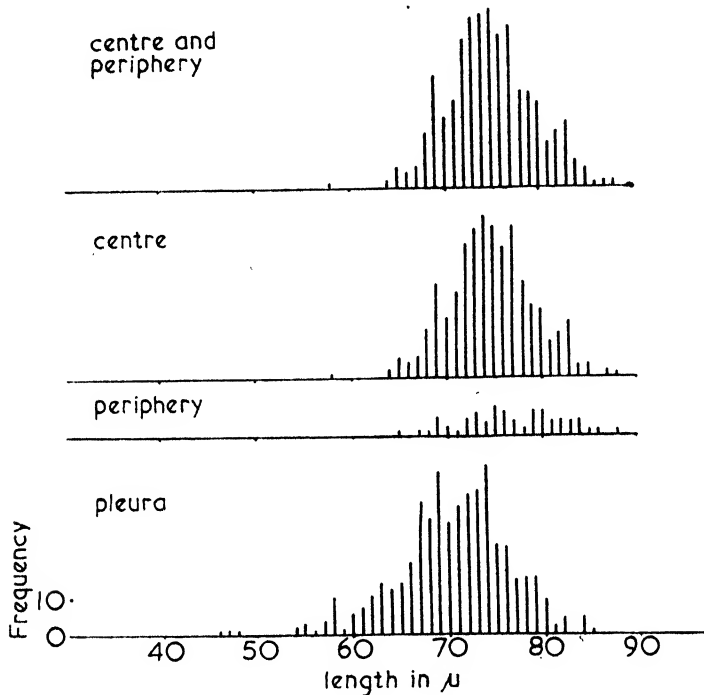


FIG. 2. Frequency-distribution curves (a) of the lengths of larvae at the centre and at the periphery of a thin blood film, fixed while wet in hot formol-Zenker's solution, showing the very small difference between the two zones ; and (b) of the lengths of larvae in the pleura, showing a number of short forms and a difference in average length between the pleural forms and those in the blood.

vitelline membrane occurring in the earlier stages, is not normally present, although it has occasionally been observed.

The most advanced stages of larval development were, of course, found in the blood, where for the first time the larva clearly revealed the primordia of the adult. It is usual to consider the presence or absence of a sheath as a constant feature of certain species, and, though this is certainly true of *Acanthocheilonema perstans*, it is not invariably so either in the case of *Loa loa* and *Wuchereria bancrofti*, where unsheathed larvae are not uncommon, or in the case of *L. carinii*, where the variability is even more marked. A sheath can usually be demonstrated in more than 50 per cent. of *L. carinii* larvae, and it may possibly be present in an even larger percentage, though, perhaps because it has not

TABLE II

Comparison of the measurements of larvae at the centre and at the periphery of thin films made from the blood with those made from the pleura, fixed whilst wet in formol-Zenker's solution. (Cotton rat 100, December 2nd, 1947)

Larvae in pleura		Larvae in peripheral blood		
Length, in μ	No.	Length, in μ	No. at periphery of film	No. at centre of film
46	1	58		1
47	1	64		2
48	1	65	1	5
54	2	66	0	4
55	3	67	1	5
56	1	68	1	13
57	3	69	4	25
58	10	70	2	16
59	1	71	1	22
60	6	72	4	35
61	8	73	6	39
62	11	74	3	43
63	14	75	7	40
64	12	76	6	34
65	14	77	3	40
66	19	78	1	25
67	35	79	6	19
68	31	80	6	17
69	43	81	3	9
70	30	82	3	12
71	34	83	3	14
72	37	84	4	3
73	38	85	1	3
74	45	86	1	0
75	29	87	0	2
76	28	88	1	1
77	14	90	0	2
78	15			
79	15			
80	9			
81	2			
82	4			
84	4			
85	1			
97	1			
Mean length	70.4789 μ		76.65 μ	74.83 μ
No. of observations ...	522		68	431
Sum sq. from mean ...	18,420.27 μ^2		1,735.53 μ^2	8,668.64 μ^2
Estimated variance of mean	0.067731 μ^2		0.3809 μ^2	0.0468 μ^2
		Difference of means		= 1.82 μ
		Estimated variance of difference		= 0.4277 μ^2
		Estimated standard error of difference		= 0.654 μ

The mean for the centre of the blood film (74.83 μ) is nearer that of the pleural film (70.4789 μ) than is the mean for the periphery (76.65 μ). Hence, taking the unfavourable case,

$$\begin{aligned}
 \text{Difference of means} &= 4.35\mu \\
 \text{Estimated variance of difference} &= 0.1145\mu^2 \\
 \text{Estimated standard error of difference} &= 0.3384\mu
 \end{aligned}$$

separated from the underlying cuticle or because it is too closely applied to the larva to be demonstrable, we have not been able to prove it so. Accompanying the morphological changes which have been shown to occur in the larva in its progress from the vicinity of the parent first to the pleura and finally to the peripheral blood, there is a slight but

steady increase in length. Once in the peripheral blood, however, development in size and morphology seems to be in abeyance, for no further change appears to occur until the larva is ingested by the vector mite.

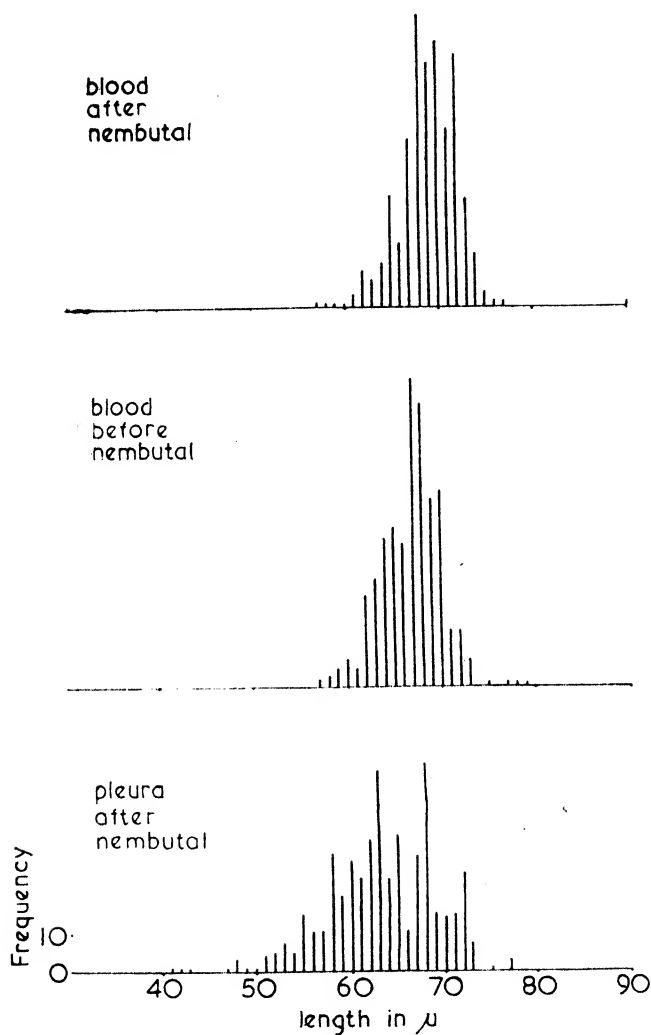


FIG. 3. Frequency-distribution curves (a) of the lengths of larvae in the blood before and after the administration of nembutal, showing an increase in length after nembutal had been administered; and (b) of the lengths of larvae in the pleura after nembutal, showing that, despite the drug, the average length is less in the pleural forms than in the blood forms.

DISCUSSION

From these results it is evident that the development of the early stages of the larva of *L. carinii* is a continuous process from birth to the final site in the peripheral blood. This being so, it is profitable to compare this development with such corresponding stages as are known in *W. bancrofti* and *L. loa*. In the case of *L. carinii* it has been shown that the

TABLE III

Comparison of the measurements of larvae in the blood before and after the administration of nembutal with those from the pleura after the administration of nembutal. (Thin films fixed whilst wet in formol-Zenker's solution. Cotton rat 147, January 3rd, 1948)

Larvae in pleura		Larvae in peripheral blood		
Length, in μ	No.	Length, in μ	No. before nembutal	No. after nembutal
31	1	57	2	2
41	1	58	3	1
42	1	59	5	1
43	1	60	8	1
47	1	61	5	3
48	3	62	24	10
49	1	63	29	7
50	1	64	40	13
51	4	65	43	21
52	5	66	38	17
53	8	67	82	45
54	6	68	76	77
55	16	69	50	64
56	11	70	53	71
57	11	71	16	48
58	32	72	15	68
59	20	73	7	29
60	29	74		14
61	24	75	1	4
62	35	76		2
63	53	77	1	2
64	24	78	1	
65	36	79	1	
66	11			
67	31			
68	55			
69	15			
70	14			
71	15			
72	26			
73	8			
75	1			
77	3			
Mean length ...	63.2048 μ		66.8080 μ	69.1080 μ
No. of observations ...	503		500	500
Sum sq. from mean ...	17,523.91 μ^2		5,015.57 μ^2	4,648.17 μ^2
Estimated variance of mean	0.069400 μ^2		0.020102 μ^2	0.018630 μ^2

Pleura versus blood before nembutal :

Difference of means = 3.61 μ
 Estimated variance of difference = 0.0895 μ^2
 Estimated standard error of difference = 0.299 μ

Pleura versus blood after nembutal :

Difference of means = 5.91 μ
 Estimated variance of difference = 0.0880 μ^2
 Estimated standard error of difference = 0.297 μ

adults are capable of direct examination, and that an additional opportunity is provided in the pleura of observing an intermediate stage in its migration to the blood-stream, whereas in *W. bancrofti* and *L. loa* the only stage about which there is incontrovertible information is that of the final fully developed larva in the blood, with its uniform morphology. Other earlier stages have been described by various authors, but their

descriptions are open to the criticism that the indirect methods which of necessity they have employed to examine the larvae in their migratory phases, or at the time of release from the adults, may have produced appearances unlike those which would be seen in their undisturbed natural state. Yet, despite this handicap, it is of interest to compare the known early larval development of *L. carinii* with the less clearly defined development of the larvae of *W. bancrofti* and *L. loa*.

It would appear from the literature that the larval development of the human filariae has been considered in two stages, instead of in the three stages of *L. carinii*, for no distinction has been drawn between those forms seen issuing from the parent female and those which have been found later in their migratory phase. Perhaps this is due to difficulty in obtaining any reliable information on the birth of the larvae of *W. bancrofti* and *L. loa*, despite the direct observations that have been made on the larval forms born from the

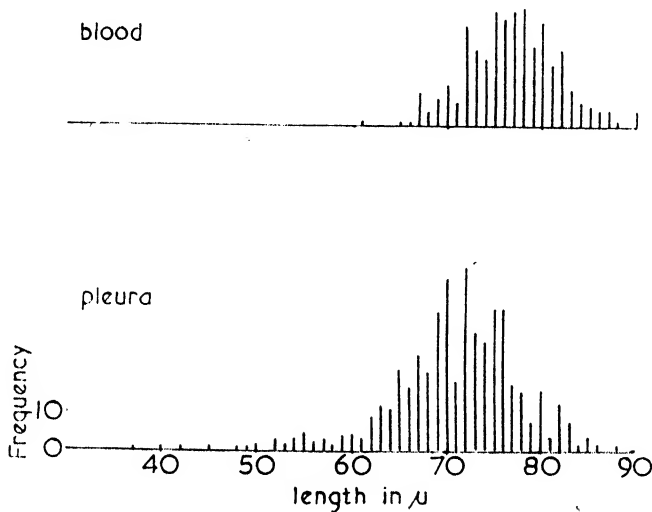


FIG. 4. Frequency-distribution curves of the lengths of larvae in the blood and in the pleura, showing a difference in average length and the presence of short forms in the pleura.

adult females of both species, for, in order to make such observations, the adults must be removed from their normal environment. Again, in *L. loa* there is no opportunity of examining the migrating larva after its release in the connective tissue, though in *W. bancrofti* it is easy to obtain this intermediate stage in the lymph-channels. It is convenient, therefore, to consider together first the direct observations on the birth of the larvae of both organisms, and later the results of indirect examination of the migratory larvae of *W. bancrofti*.

Bahr (1912) watched the emergence of great numbers of extended larvae, enclosed in their sheaths, from the vagina of an intact female *W. bancrofti*, but saw no coiled embryos passed. On placing fragments of a gravid female in normal saline, he saw, streaming from the uterine tubes, ova containing coiled embryos which, when the slide was warmed, became extended and stretched their chorionic envelopes to form sheaths. Roy (1923) found eggs, some of which were undergoing degenerative changes, and larvae, in various

TABLE IV

Comparison of the measurements of larvae in the blood with those in the pleura. (Thin films fixed whilst wet in formol-Zenker's solution. Cotton rat 25, February 28th, 1948)

Larvae in pleura		Larvae in peripheral blood	
Length, in μ	No.	Length, in μ	No.
37	1	61	1
40	1	65	1
42	1	66	1
45	1	67	8
48	1	68	3
49	1	69	7
50	2	70	11
52	3	71	6
53	2	72	27
54	3	73	20
55	4	74	18
56	2	75	29
57	3	76	28
58	1	77	29
59	3	78	31
60	4	79	22
61	3	80	27
62	9	81	16
63	12	82	20
64	11	83	9
65	22	84	6
66	17	85	5
67	26	86	3
68	21	87	3
69	37	88	1
70	46	90	3
71	18		
72	48		
73	31		
74	29		
75	38		
76	38		
77	18		
78	16		
79	7		
80	16		
81	3		
82	12		
83	8		
84	1		
85	3		
88	1		
Mean length ...	70.8492 μ		76.6806 μ
No. of observations ...	524		335
Sum sq. from mean ...	24,153.1 μ^2		7,260.82 μ^2
Estimated variance of mean	0.088133 μ^2		0.064892 μ^2

$$\begin{aligned}
 \text{Difference of means} &= 76.68 - 70.85 = 5.83\mu \\
 \text{Estimated variance of difference} &= 0.153025\mu^2 \\
 \text{Estimated standard error of difference} &= 0.3911\mu
 \end{aligned}$$

stages of development, in the urine of two cases of chyluria and from the lymph in a case of lymph-scrotum. Hegner *et al.* (1938) observed that in *W. bancrofti* the vitelline membrane can be seen clearly round the egg, but with difficulty round the larva about to be born, and maintained that hatching occurs either in the uterus or shortly after birth.

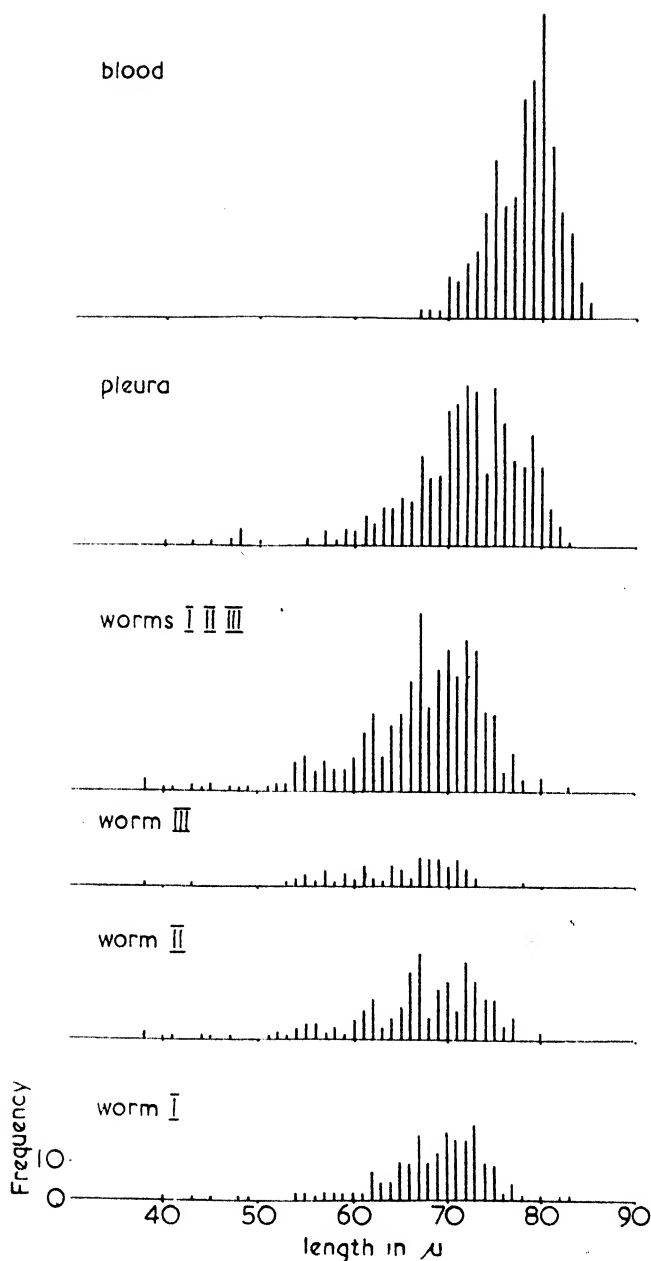


FIG. 5. Frequency-distribution curves (a) of the lengths of larvae taken from the immediate neighbourhood of adult females (worms I, II and III), showing the presence of short forms and the very small difference in the average length; (b) of the lengths of larvae taken from worms I, II and III, from the pleural exudate and from the blood, showing a progressive increase in average length in the larvae from the three sites. Short forms are to be seen in the pleura, but the blood forms are shown to be more uniform, and the steepness of the margin on the right indicates that they have reached a stable form with a maximum length.

TABLE V

Comparison of the measurements of larvae from the immediate neighbourhood of three worms, with those in the pleural exudate and the blood. (Thin films fixed whilst wet in formol-Zenker's solution. Cotton rat 152, April 3rd, 1948)

Larvae					Larvae in peripheral blood	
Length, in μ	No. near worm I	No. near worm II	No. near worm III	No. in pleura	Length, in μ	No.
38		2	1		67	2
40		1		1	68	2
41		1			69	2
43	1		1	1	70	11
44		1			71	9
45	1	1		1	72	14
47		1		1	73	18
48	1			4	74	28
49	1				75	42
50				1	76	29
51		1			77	32
52		2			78	58
53		1	1		79	63
54	2	3	2		80	80
55	2	4	3	2	81	45
56	1	4	1		82	28
57	2	2	4	4	83	23
58	2	3	1	1	84	9
59	2	1	3	4	85	4
60	2	5	2	3	90	1
61	2	8	6	7		
62	8	11	2	6		
63	5	3	1	10		
64	5	6	6	10		
65	9	8	4	13		
66	9	18	2	12		
67	17	23	7	24		
68	10	6	7	17		
69	16	13	7	18		
70	18	15	5	36		
71	16	8	7	37		
72	16	21	4	43		
73	21	15	2	41		
74	10	11		19		
75	9	11		42		
76	2	3		33		
77	5	5		22		
78	2		1	21		
79				29		
80	1	2		21		
81				11		
82	1			5		
83	1			1		
Mean length ...	68.5800 μ	66.7636 μ	64.5250 μ	71.694 μ	77.932 μ	
No. of observations	200	220	80	500	500	
Estimated variance of mean ...	0.183183 μ^2	0.251841 μ^2	0.573409 μ^2	0.0819567 μ^2	0.0246480 μ^2	

Worm films : 1 (I) — 2 (II) + 1 (III) = -0.4222 μ
 Variance = 1.763956 μ^2

1 (I) — 1 (III) = 4.0550 μ
 Variance = 0.756592 μ^2
 $\chi^2 = 21.733$

$\chi^2 = 0.101$
 i.e., — $\chi^2 21.834$ (2 d.f.) $P < 0.001$

Pleura versus peripheral blood

Difference = 6.238 μ
 Variance = 0.1066047 μ^2
 Standard error = 0.3265 μ

Pleura versus worm I

Difference = 3.1140 μ
 Variance = 0.265140 μ^2
 Standard error = 0.5149 μ

Peripheral blood versus worm I

Difference = 9.3520 μ
 Variance = 0.207831 μ^2
 Standard error = 0.4559 μ

Huffman (1911), in establishing the identity of the embryo of *Filaria loa* and of the '*Filaria diurna*' of Manson, attempted without success to induce a gravid female *L. loa*, which had been removed from a human eye, to give birth by placing it in warm tap-water, then in blood, and finally by pressure and irritation; no embryos were present in the water in which the worm had been left overnight at 33° C. He found the uterus full of developed uncoiled embryos, packed in bundles end to end; yet on cutting the worm transversely myriads of coiled embryos in their vitelline membranes escaped, and some of them then extended and stretched their membranes.

Several indirect observations have been made in human filariasis of the early developmental stages immediately after birth, which correspond to those larvae found near the adult *L. carinii* and in the pleura. Manson (1883) aspirated fluid from varicose lymph-nodes and from vesicles in a lymph-scrotum in cases suffering from filariasis in Amoy, and described immature microfilariae and embryos coiled up in their vitelline membranes; but he assumed that at least the ova, and perhaps even the immature larval forms, were indications of miscarriage, and held that the sheath of the coiled embryos was the same as that round the larvae in the blood. Bahr (1912) obtained microfilariae on six occasions by gland puncture, once in hydrocoele fluid, and once in purulent exudate from the tunica vaginalis. In three of these the microfilariae were small, with the sheath extended anteriorly and posteriorly and with the nuclear structure still undifferentiated, closely resembling the small forms seen emerging from the vagina of the adult worm. And in two instances, in addition to microfilariae, ova and empty sheaths were found in considerable numbers. In a case of lymphangitis of the leg associated with elephantiasis, however, he failed to find any difference between those in the lymph from the gland and those in the blood.

In commenting on Manson's original findings, Daniels (1898) suggested that the genital aperture of the female, though sufficiently large to allow the extended embryo to pass, is too small to permit the passage of eggs or coiled larvae. However true this may be in the case of *W. bancrofti*, it must be borne in mind that nematodes have a far from rigid structure, and that much larger and less elastic eggs are passed quite normally by worms more slender than the filariae, the trichostrongyles passing eggs 80 μ by 40 μ , and *Nematodirus spathiger* laying an egg 200 μ by 100 μ .

It is not surprising, however, that, on other grounds, doubt has been cast on the validity of some of these earlier observations, particularly on those based on gland puncture, for the adults of *W. bancrofti* are so inextricably entwined in the tissues of the gland that the possibility of setting free the early forms, either by direct rupture or by mechanical interference with the adult, is not unlikely. These objections do not, of course, arise in the case of *L. carinii*, for in the early stages, before they provoke any fibrous reaction, the worms are motile and lie free, and are easily accessible in the open spaces of the pleural sac. Moreover, the microfilariae obtained from the immediate neighbourhood of the parent worm can be compared with those in the pleural sac and with those in the blood, additional confirmation being thus afforded that differences in form are not due in part to trauma.

It is difficult to compare the increase in length which occurs in the microfilariae of *L. carinii* with similar increases observed in the larvae of *W. bancrofti* and *L. loa*, because of the uncertainty attached to the environment in other experiments, and to the lack of sufficient data for statistical comparison. Penel (1905), Fülleborn (1929) and Bach (1913)

considered that the fresh-born larvae of *W. bancrofti* are little smaller than those in the circulation, Penel believing that the increase in size is due to swelling of the larvae which had previously been compressed in the uterus. Fantham *et al.* (1916) stated that the larvae of *W. bancrofti* measure at birth $127\text{--}200\mu$ by $8\text{--}10\mu$, and in the blood, when fresh, 260μ by $7.5\text{--}8\mu$, though they noted that in stained films, owing to shrinkage, there is a great variation in size—from 154μ to 311μ . O'Connor (1923) considered that thickness of the body and the lack of orderly arrangement of the nuclei, rather than length, were suggestive that microfilariae were new-born. In contrast—though the observations are not strictly analogous—Rogers (1920), in treating cases of *W. bancrofti* infection with tartar emetic, found that in the unsuccessful cases there was a rapid increase in the number of microfilariae immediately after the injections were stopped, accompanied by the appearance of very numerous long thin larvae, which he assumed to be younger forms.

It would appear from these accounts that the few known stages in the life-cycle of the larvae of *W. bancrofti* and *L. loa* are similar to comparable stages in the more fully studied cycle of *L. carinii*; and it seems legitimate, therefore, to postulate the probability that the life-cycle of these three larvae will be found to be similar in principle, if not in detail.

Attempts have been made in the past to induce growth of microfilariae *in vitro*, and many workers have maintained them in culture media—usually in order to study their metabolism—and have used observations on an increase in length to support a contention that active development has taken place. Wellman and Johns (1912), Bach (1913), Johns and Querens (1914) and Joyeux and Sautet (1937) all kept *Dirofilaria immitis* larvae in serum, with the addition, perhaps, of dextrose or haemolyzed blood, for 12–15 days, and observed that growth occurred in some of them. Low (1912), in criticizing the suggestion of Wellman and Johns that the increased length of the embryos corresponded to their limit of development in the digestive tract of the mosquito, maintained that, as regards measurements and dimensions, the forms seen in the stomach of the mosquito and in the blood of the definitive host are identical. Johns and Querens (1914) kept the larvae of *Setaria labiato-papillosum* (= *S. cervi*) alive in calf blood for 52 days, and found that they increased in length and thickness. Coutelen (1929) found that, while no growth or change occurred in the larvae of a frog filariid, *Icosiella neglecta*, in several media, *Microfilaria bancrofti* could be kept alive for 32 days in unheated serum, and that they grew and lost their sheaths, ecdysis occurring particularly in solutions to which normal saline had been added. The artificiality of environment inevitable in all these observations, however, does not warrant any conclusions being drawn on the possibility of growth occurring under natural conditions.

It will thus be seen that the development of the larvae of *L. carinii* is characterized by the shedding of the vitelline membrane, by the acquiring of a sheath, and by changes in length, together with certain alterations in the arrangement of the nuclei; and it is of interest to consider what importance should be attached to these changes as indications of development. The development can be followed continuously from the shedding of the vitelline membrane at birth, through an increasing complexity of nuclear architecture, with a small successive increase in length occurring with migration to the pleural sac and finally to the peripheral circulation. The less complex larvae in the pleura are unsheathed; in some of those in the peripheral circulation sheaths can be demonstrated easily, in others only with difficulty or not at all. The morphological development of

the blood forms, whether sheathed or unsheathed, is, however, uniform, and clearly the presence or absence of a sheath cannot be taken as sole evidence of maturity.

It has been the custom to attach much importance to this feature—i.e., the presence or absence of a sheath—in the larval stage of the filariae, as constituting a clear-cut indication of development; but when the life-cycle of these organisms is compared with that of the other nematodes, particularly of those which are parasitic in other sites in vertebrates and have free-living phases in their cycles, the presence or absence of a sheath becomes of less importance than the other aspects of their morphological development. Thus, in some nematode larvae the newly acquired sheath may separate and be shed immediately, in others it may be retained for some time, while in the case of *Dictyocaulus filaria*, which occurs in the bronchi of sheep, the first larval sheath is not cast until after the second has separated, so that the free-living third-stage larva is enclosed for some time in two separate larval skins. So far as the sheath is concerned, larval metamorphosis includes three processes: the formation of the sheath by the production of a new cuticle to replace it, grown by the subcuticular layer beneath; its separation from the new surface of the larva; and finally its rupture and abandonment, or ecdysis. Of these cuticular changes the most significant is the formation of the sheath, since this is more closely related to the stage of development than either the separation or the ecdysis; it does not follow, however, that the formation of the sheath necessarily coincides with the much more vital physiological demarcation between two stages in development.

However this may be, the failure to demonstrate the presence of a sheath must not be taken as evidence of maturity—a point well demonstrated in the case of the larvae of *L. carinii*. If a thick blood film containing larvae of this species is examined, although all the larvae at the periphery show a separate sheath, many of those at the centre do not, and, even in those in which it can be seen, it is much less obvious than in those at the periphery. Whether this separation is due to a difference in the osmotic tension in the two zones of the drying film, or to a difference in the mechanical fixation of the sheath by the drying blood and its separation by the movement of the larva, or to a combination of both factors, is immaterial to the argument; the fact remains that the sheath has been formed and is ready to separate when the proper conditions are satisfied.

It would appear, then, that the larvae of *L. carinii* in the peripheral blood all possess a sheath, though in many cases it has not separated from the cuticle. The casting of this sheath, as in other microfilariae, does not occur until ingestion by the vector, and this ecdysis occurs almost immediately after ingestion by *L. bacoti*, for Bertram *et al.* (1946) counted over 100 empty sheaths in the changed blood in the gut of the adult female mite within an hour of its having begun to feed on an infected cotton rat. Lapage (1937) has shown that the factors precipitating the ecdysis in the second larval stage of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus* spp. are changes in the pH and in the osmotic tension of their environment; and no doubt similar changes occur in the case of *L. carinii*, both at its birth and again on ingestion by the vector.

The most striking events in the development of the early forms of *L. carinii* are the shedding of the vitelline membrane, which liberates the first larval stage in the pleura of the rat, and the ecdysis of the first larval sheath in the gut of the mite. The microfilaria in the peripheral circulation of the rat should, therefore, irrespective of whether or not it has developed a sheath, be regarded as a first-stage larva, corresponding to the first-stage larva of the parasitic intestinal nematodes of vertebrates.

SUMMARY

1. The advantages are discussed of the use of the cotton rat infected with *Litomosoides carinii* as an experimental infection in the chemotherapy of filariasis, and the necessity is stressed for more detailed knowledge of the development of the early larval stages.

2. A method is described for obtaining larvae at the following three stages in their migration: from the neighbourhood of the adult female on the mediastinum, from the general pleural space, and from the peripheral blood.

3. A technique for the measurement of the length of the larvae obtained under standard conditions from the three sites is described.

4. The larval development is followed from birth, through migration in the pleural cavity, to the peripheral circulation, and is shown to be characterized by the shedding of the vitelline membrane at birth, through an increasing complexity of nuclear structure, with a gradual increase in length, until a stable form is attained, with uniform morphology and maximum length, in the peripheral circulation.

5. The significance of these changes is discussed, and the importance is stressed of the shedding of the vitelline membrane on the change of environment accompanying birth and the abandonment of the first larval sheath in the gut of the insect vector. The microfilaria in the peripheral circulation is identified as the first-stage larva, corresponding to the first-stage larva of the parasitic intestinal nematodes.

APPENDIX

STATISTICAL ANALYSIS OF THE DATA

by

R. L. PLACKETT

For any sample let x be length in microns, n the number of microfilariae of this length, and N the total number in the sample. Using S to denote summation over the sample, the sample mean is

$$\bar{x} = (S n x)/N$$

the variance of the hypothetical population whence the sample is drawn is estimated as

$$s^2 = [S n x^2 - (S n x)^2/N]/(N-1)$$

and the estimated variance of the sample mean is s^2/N .

In calculating these quantities it is often convenient to use a working mean, namely, a value of x in the neighbourhood where observations are most frequent; if this is, for example, 77μ , then 77 is subtracted from each observation, so that 71 becomes -6 and $88 + 11$. The mean and variance are calculated from the adjusted observations, which are now much smaller and easy to manipulate; the mean of the original observations is got by adding 77 to the adjusted mean, while the variance is unaltered. This procedure is described in any text-book on applied statistics; see, for example, Fisher (1946), § 13.

Now let there be two samples to compare. The difference of sample means is $x_1 - \bar{x}_2$ and the estimated variance of this quantity is $s_1^2/N_1 + s_2^2/N_2$; it is further necessary to calculate the estimated standard error of $x_1 - \bar{x}_2$, namely $\sqrt{[s_1^2/N_1 + s_2^2/N_2]}$. Values of t , the ratio of $x_1 - \bar{x}_2$ to its estimated standard error, which are in excess of 3 may be regarded as significant evidence that the samples were drawn from populations with different mean values; this criterion is satisfied in all except one of the sample pairs

examined, t being usually much greater than 3; the exceptional value, 2.78, is also sufficiently large to draw the same conclusion.

In one instance there are three samples to be compared. Denoting these arbitrarily by 1, 2 and 3, calculate

$$\frac{(\bar{x}_1 - 2\bar{x}_2 + \bar{x}_3)^2}{\left(\frac{s_1^2}{N_1} + \frac{4s_2^2}{N_2} + \frac{s_3^2}{N_3}\right)} + \frac{(\bar{x}_1 - \bar{x}_3)^2}{\left(\frac{s_1^2}{N_1} + \frac{s_3^2}{N_3}\right)}$$

To test the significance of this quantity, refer it to a table of χ^2 with 2 degrees of freedom; in our example $\chi^2 = 21.834$, a highly significant result occurring by chance in less than one in 1,000 experiments if no differences existed between the three population means.

It should be emphasized that the methods given are valid only if the sample sizes N are sufficiently large; while this condition is satisfied here, other techniques and additional assumptions are required from smaller N .

To proceed to more detail, the difference in length between the centre and periphery of thick film T of cotton rat 132 is 16.96μ , estimated standard error 2.154μ , $t = 7.87$; for thick film L₁ the corresponding figures are 16.33μ , 1.655μ and 9.87 ; for thick film L₂ 9.58μ , 1.888μ and 5.07 . All these differences are highly significant.

For cotton rat 100, the difference between the centre and periphery of the blood film is 1.82μ , standard error 0.654μ , $t = 2.78$. This indicates that the microfilariae in the centre are on the average shorter; they therefore provide the unfavourable case in a comparison with the pleural film attempting to demonstrate the smaller pleural mean. Making this comparison, the mean difference between the pleural film and centre of the blood film is 4.35μ , standard error 0.338μ , $t = 12.9$.

For cotton rat 147, there are two blood films, one before and the other after the administration of nembutal. In the absence of nembutal, the difference between the means of the pleura and blood is 3.61μ , standard error 0.299μ , $t = 12.1$; with nembutal the corresponding figures are 5.91μ , 0.297μ , $t = 19.9$.

For cotton rat 25, the difference between the means of pleura and blood is 5.83μ , standard error 0.391μ , $t = 14.9$.

For cotton rat 152, the three worm films have means of 68.58μ , 66.76μ and 64.52μ ; and the estimated variances of these means are respectively $0.1832\mu^2$, $0.2518\mu^2$ and $0.5734\mu^2$. Calculating χ^2 as indicated, we find $\chi^2 = 21.8$ on 2 degrees of freedom, corresponding to a probability P less than 0.001. Here the most unfavourable comparison with pleural and peripheral films is given by the first worm film: pleura versus worm I yields a mean difference of 3.11μ , standard error 0.515μ , $t = 6.04$; for peripheral blood versus worm I the corresponding figures are 9.35μ , 0.456μ , $t = 20.5$.

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AUTHOR INDEX

- Adler, S., 1, 178
 Andrews, W. H. H., 95
 Bach, L. G., 153
 Baz, I., 304
 Ber, M., 1
 Black, R. H., 52, 88, 90, 236
 Blair, D. M., 46
 Carter, H. F., 312
 Chwatt, L. J., 101, 184, 251, 364
 Coghill, N. F., 113, 288
 Corkill, N. L., 224, 230, 278
 Cowper, S. G., 119
 Crewe, W., 334
 Damkas, C., 153
 Davis, D. H. S., 207
 Dax, R., 153
 Depanian, M., 153
 Dick, G. W. A., 218, 271
 Fairbairn, H., 118
 Foy, H., 153
 Friedheim, E. A. H., 357
 Gall, D., 54
 Gambles, R. M., 113, 288
 Gönnert, R., 256
 Gordon, R. M., 251, 334, 364
 Gramiccia, G., 88
 Haddow, A. J., 218, 271
 Halawani, A., 304
 Jones, C. M., 251, 364
 Jones, W. R., 33
 Kershaw, W. E., 377
 Kettle, D. S., 5
 Kikuth, W., 256
 Kirk, R., 322
 Kitchen, S. F., 218
 Kondi, A., 153
 Langton, M., 153
 Leeson, H. S., 253
 Lefcopoulou, T., 153
 Lewis, D. J., 322
 Lourie, E. M., 241
 Lumsden, W. H. R., 218
 Macfie, J. W. S., 67
 Maegraith, B. G., 95
 Minning, W., 268
 Morcos, F., 304
 Nash, T. A. M., 30
 Pawan, J. L., 173
 Pitchford, J., 153
 Plackett, R. L., 377
 Rollo, I. M., 241
 Ross, W. F., 46
 Shiele, P., 153
 Smithburn, K. C., 218
 Spinks, A., 190
 Stewart, G. T., 33, 198
 Tchernomoretz, I., 1
 Unsworth, K., 249
 Vanderplank, F. L., 131
 Vogel, H., 268
 Williamson, J., 238, 241
 Zuckerman, A., 178

SUBJECT INDEX

- Aedes (Aedimorphus) boneti* s.sp. *kumbae* s.sp. nov., a new *Aedes* from the Cameroons, 184
Aedes (Stegomyia) africanus Theobald, implication of, in the forest cycle of yellow fever in Uganda, 218
 Amoebiasis, intestinal, in man, the rôle of bacteria in, 198
 Amoebic infection in the rat, experimental, pathology of, 33
Anopheles sergenti, growth of, with special reference to the growth of the anal papillae in varying salinities, 5
 Anopheline larvae collected in Arabia, 253
 Antimalarial drugs, field trials with new anti-malarial drugs in Egypt, 304
 Arabia, anopheline larvae collected in, 253
 Bacteria, the rôle of, in intestinal amoebiasis in man, 198
 Bats, fruit-eating, and paralytic rabies in Trinidad, 173
 Blackwater fever and malaria in Macedonia and Thrace in relation to DDT, 153
 Cameroons, a new *Aedes* from, 184
 Carbon tetrachloride, pathogenesis of the liver lesion due to the administration of, 95
 Cercarial antigen, the use of, in the diagnosis of schistosomiasis, 46
 Ceylon, records of filaria infections in mosquitoes in, 312
Chrysops, observations on the feeding of, 334
Chrysops sp., breeding-places of, 251, 364

- Cimex*, observations on the feeding of, 334
Culicoides, some species of, from the State of Chiapas, Mexico, 67
Cyprus, relapsing fever in, 288
- DDT, malaria and blackwater fever in Macedonia and Thrace in relation to, 153
Diamidines, action *in vitro* of some aromatic diamidines on a Sudan strain of *Leishmania infantum*, 1
Dioscorea dumetorum Pax as a famine food in the Anglo-Egyptian Sudan, 278
Diptera, catching of biting Diptera in Uganda, with anaesthetized monkeys as bait, 271
- Egypt, field trials with new antimalarial drugs in, 304
Entopoloypoides macaci, occurrence of, in East African monkeys, 118
Exoerythrocytic forms of *Plasmodium gallinaceum*, cultivation of, 88
- Filaria infections in mosquitoes in Ceylon, 312
- Glossina*, effect of high temperature on the pupal stage of, in relation to the transmission-rate of trypanosomes, 30
Glossina, experiments in cross-breeding, 131
- Haemoglobin, resorption of, by the renal tubules in haemoglobinuria, 90
Haemoglobin stains adapted for use with thin blood films, 236
- Kala-azar, latent, activation of, by malaria and relapsing fever, 230
Kala-azar and malaria, latent, activation of, by battle experience, 224
Kumba, British Cameroons, survey of loiasis at, with description of the breeding-places of the vector and suggestions for future research and possible methods of control, 364
- Leishman's stain adapted for use with histological sections, 52
Leishmania infantum, action *in vitro* of some aromatic diamidines on a Sudan strain of, 1
Leishmania tropica, observations on a strain of, after prolonged cultivation, 178
Litomosoides carinii, development of the first-stage larva, 377
Liver lesion due to the administration of carbon tetrachloride, pathogenesis of, 95
Loiasis, survey of, at Kumba, British Cameroons, with description of the breeding-places of the vector and suggestions for future research and possible methods of control, 364
- Macedonia and Thrace, malaria and blackwater fever in, in relation to DDT, 153
Malaria, infection of reticulocytes by *P. falciparum* and *P. malariae* in hyperendemic indigenous malaria, 101
- Malaria and blackwater fever in Macedonia and Thrace in relation to DDT, 153
Malaria and kala-azar, latent, activation of, by battle experience, 224
Malaria and relapsing fever, activation of latent kala-azar by, 230
Melarsen oxide in the treatment of human trypanosomiasis, 357
Mexico, some species of *Culicoides* from the State of Chiapas, 67
Microscopy of living tissues *in situ*, a simple technique for, 54
Miracid, action of, in *Schistosoma japonicum* infections in laboratory animals, 268
Mosquitoes, mechanisms by which mosquitoes and tsetse-flies obtain their blood-meal, histology of the lesions produced, at subsequent reactions of the mammalian host, 334
Mosquitoes, records of filaria infections in mosquitoes in Ceylon, 312
- Nigeria, occurrence of larvae of *Oestrus ovis* in the nasal cavities and frontal sinuses of goats in, 249
Nomogram for the preparation of standard inocula in the Davey chick test of antimalarial activity, 238
- Oestrus ovis*, occurrence of larvae of, in the nasal cavities and frontal sinuses of goats in Nigeria, 249
- Paludrine, blood concentrations and physiological distribution of some homologues of paludrine in relation to their antimalarial activities, 190
Paludrine, failure to produce resistance to, in *Plasmodium gallinaceum* by prolonged treatment of latent infections, 241
Phlebotomus, taxonomy of the Ethiopian sand-flies, 322
Plague, sylvatic, in South Africa: history of plague in man, 1919-43, 207
Planorbis corneus, effect of certain inorganic and vegetable substances on, 119
Plasmodium falciparum and *P. malariae*, infection of reticulocytes by, in hyperendemic indigenous malaria, 101
Plasmodium gallinaceum, cultivation of exoerythrocytic forms of, 88
Plasmodium gallinaceum, failure to produce paludrine-resistance in, by prolonged treatment of latent infections, 241
- Rabies, paralytic, and fruit-eating bats in Trinidad, 173
Relapsing fever, methods for differentiating tick- from louse-borne spirochaetes, 113
Relapsing fever and malaria, activation of latent kala-azar by, 230
Relapsing fever in Cyprus, 288
Reticulocytes, infection of, by *P. falciparum* and *P. malariae* in hyperendemic indigenous malaria, 101

- landflies (*Phlebotomus*), taxonomy of the Ethiopian, 322
- Leishmania japonicum* infections in laboratory animals, action of miracid in, 268
- Leishmaniasis, the use of cercarial antigen in the diagnosis of, 46
- Leishmaniasis, experimental studies on the therapy of, 256
- South Africa, sylvatic plague in: history of plague in man, 1919-43, 207
- Leishmaniasis, relapsing fever, methods for differentiating tick- from louse-borne, 113
- Leishmaniasis, splenic circulation, some observations on, 54
- Uganda, the poisonous wild cluster yam, *Dioscorea dumetorum* Pax, as a famine food in, 278
- Thrace and Macedonia, malaria and blackwater fever in, in relation to DDT, 153
- Trinidad, fruit-eating bats and paralytic rabies in, 173
- Trypanosomes, effect of high temperature on the pupal stage of *Glossina* in relation to the transmission-rate of, 30
- Trypanosomiasis, human, melarsen oxide in the treatment of, 357
- Tsetse-flies, mechanisms by which mosquitoes and tsetse-flies obtain their blood-meal, histology of the lesions produced, and subsequent reactions of the mammalian host, 334
- Uganda, catches of biting Diptera in, with anaesthetized monkeys as bait, 271
- Uganda, implication of *Aedes (Stegomyia) africanus* Theobald in the forest cycle of yellow fever in, 218
- Yam, the poisonous wild cluster yam as a famine food in the Anglo-Egyptian Sudan, 278
- Yellow fever in Uganda, implication of *Aedes (Stegomyia) africanus* Theobald in the forest cycle of, 218

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